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13. ABSTRACT ***(Maximum 200 Words)***

For a drug that is used so widely in breast cancer therapy, little is known about the mechanism of Taxol action. Although Taxol's binding to tubulin is well characterized and the ability of the drug to induce a mitotic arrest is recognized, the biochemical mechanisms by which these events lead to breast cancer cell cytotoxicity are not known. The objective of this research is to test the hypothesis that prolonged activation of Cdc2 and subsequent phosphorylation of Bcl2 are required for Taxol-mediated apoptosis in breast cancer cells. The ultimate goal of the research is to identify a signaling cascade(s) that is important for the cytotoxic effects of Taxol. Progress to date includes: (i) development of cell model systems to analyze the role of Bcl2 phosphorylation in susceptibility to Taxol-mediated apoptosis; (ii) determining that Bcl2 overexpression provides a selective advantage for cell survival and growth in anchorage-independent settings *in vitro* and *in vivo*; and (iii) preliminary confirmation that Taxol-induced modulation of mitotic events that is observed in cell culture models is operational *in vivo*. It is critical to understand how Taxol works at the molecular level in order to pursue rational design of new drugs for the treatment of breast cancer.

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INTRODUCTION

For a drug that is used so widely in breast cancer therapy, remarkably little is known about the mechanism of Taxol action. Although Taxol's binding to tubulin is well characterized and the ability of the drug to induce a mitotic arrest is recognized, the biochemical mechanisms by which these events lead to breast cancer cell cytotoxicity after drug administration are not known. A number of cellular signalling pathways have been associated with Taxol's antitumor effects; however, there is a lack of studies that have focused on the role of biochemical pathways activated by Taxol treatment during mitosis, the phase of the cell cycle in which Taxol-treated cells arrest. Knowledge of mitotic signalling pathways that are altered after Taxol treatment of breast cancer cells is critical for our understanding of the drug's mechanism of antitumor action and is the focus of the proposed research. Studies from our laboratory indicate that the anti-apoptotic Bcl2 protein is normally phosphorylated during mitosis and that Taxol treatment of breast cancer cells leads to prolonged mitotic arrest, superphysiologic levels of mitotic kinase activity, and subsequent hyperphosphorylation of Bcl2. Following these events, apoptosis is initiated. Thus, our results provide a link between Taxol-induced elevation of mitotic kinase activity and the apoptotic machinery in the cell. We predict that mitotic cyclin B1/Cdc2 activity is integral for Taxol's cytotoxic activity even though we have found that it is not the kinase directly responsible for mitotic phosphorylation of Bcl2. ***We hypothesize that prolonged activation of Cdc2 and subsequent phosphorylation of Bcl2 are required for Taxol-mediated apoptosis in breast cancer cells.*** We are testing this hypothesis through the specific aims listed below. The ultimate goal of these aims is to identify a signaling cascade that is important for the cytotoxic effects of Taxol and determine if this signalling cascade is operational *in vivo* in breast carcinomas treated with Taxol.

- **Specific Aim 1:** To determine whether Cdc2 activity and Bcl2 phosphorylation are required for Taxol-mediated apoptosis.
- **Specific Aim 2:** To identify the mitotic kinase(s) that phosphorylates Bcl2 in Taxol-treated breast cancer cells
- **Specific Aim 3:** To determine if the Taxol-induced modulation of mitotic events that are observed in cell culture models is operational *in vivo* in xenograft tumors established from breast cancer cell lines

Understanding the link between Taxol modulation of microtubule dynamics, mitotic block, and cell death promises to lead to improved clinical use of the drug in the treatment of breast cancer. It is critical to understand how Taxol works at the molecular level in order to pursue rational design of new drugs for breast cancer that act through alteration of mitotic signalling pathways.

BODY

A description of the research accomplishments associated with each Task outlined in the approved Statement of Work is provided below.

Statement of Work

Task 1: **To determine whether Cdc2 activity and Bcl2 phosphorylation are required for Taxol-mediated apoptosis (months 1-12)**

•Using flow cytometric, cell biology, and protein biochemistry techniques, we determined the kinetics of mitotic entry and metaphase transition in FT210 cells and thatCdc2 was required for Taxol-mediated apoptosis once cells had entered mitosis (months 1-12).

Presented in last year's progress report.

•Using molecular biology, cell biology, and protein biochemistry techniques, we overexpressed nonphosphorylatable Bcl2 protein in breast cancer cells and determined the effect protein expression has on mitotic events and susceptibility to Taxol mediated apoptosis (months 12-24)

Progress is presented in the attached manuscript (that has been submitted): "Ectopic Expression of Wild-Type Bcl-2, not S70A Phosphomutant Bcl-2, Confers Increased Cell Survival after Transient Taxol Treatment."

A key accomplishment under this Task was the development of a 2D isoelectric focusing assay to analyze Bcl2 phosphorylation status in protein lysates prepared from tumor specimens

•Taxol-mediated phosphorylation of p53 in epithelial tumor cells

Progress presented in attached publication entitled: "Increased p53 phosphorylation after microtubule disruption is mediated in a microtubule inhibitor- and cell-specific manner."

Task 2: **To identify the mitotic kinase(s) that phosphorylates Bcl2 in Taxol-treated breast cancer cells (months 13-36)**

•Large scale protein preparation and column chromatography will be performed accompanied by enzyme activity assays (months 25-36)
•Peptide sequencing of purified protein-(s), cDNA cloning, and further characterization of gene and protein will be performed (e.g. initiate antibody production, analyze cell cycle regulation of candidate kinase) (months 25-36)

To date we have constructed the recombinant Bcl2 expression vectors and begun Bcl2 protein scale-up for column preparation. We have found that production of recombinant Bcl2 in *E. Coli* gives a very low yield due to the fact that most of the protein is present in occlusion bodies. Currently, we are contemplating switching to a baculovirus expression system. Nonetheless, we have produced enough Bcl2 to begin small-scale testing of protein extracts from Taxol-treated MDA-MB468 cells. In the grant we also proposed to use FT210 cells since they grow in suspension to high density and therefore the cost of large scale expansion would be relatively moderate. However, for the reasons discussed in our first progress report, we are refraining from using the FT210 cells until we clarify the clonality issue. To date, we have made small glutathione resin columns that have GST-Bcl2

attached. After column preparation, we introduced protein extracts from control or Taxol-treated MDA-MB468 cells onto the column, washed with physiological buffer conditions, eluted supposed Bcl2-bound proteins with a high salt, and examined the profile of proteins (by PAGE followed by silver staining) that were bound to the Bcl2. Our findings after our first trials, suggest that the protein profiles are different from control extracts as compared to those from Taxol-treated cells. We will use mass spectrometry for mass determination as well as peptide sequencing for identification of the proteins that are bound to the recombinant Bcl2.

Mass Spectrometric analyses of Bcl2 from tumor tissue and indentification of any associated proteins will be the primary focus of the remaining year of the grant.

Task 3: **To determine if the Taxol-induced modulation of mitotic events that are observed in cell culture models is operational *in vivo* in xenograft tumors established from breast cancer cell lines (months 24-36).**

•Initiate breast cancer cell line xenograft tumors in nude mice.
We were able to to generate the most reproducible data with the RKO cell line and this has been the focus of our studies. Data generated from the experiments with RKO cells is presented in the attached manuscript entitled: "Ectopic Expression of Wild-Type Bcl-2, not S70A Phosphomutant Bcl-2, Confers Increased Cell Survival after Transient Taxol Treatment."

•Taxol treatment and analysis of tumor volume as well as flow cytometric analysis of tumors

Progress on analysis of the tumor volumes is presented in the attached manuscript entitled: "Ectopic Expression of Wild-Type Bcl-2, not S70A Phosphomutant Bcl-2, Confers Increased Cell Survival after Transient Taxol Treatment."

Flow cytometric analyses were not fruitful since we were never able to get single cell suspensions of xenograft tumor cells for flow cytometry.

•Immunohistochemical and protein biochemical analysis of tumor samples
In progress

KEY RESEARCH ACCOMPLISHMENTS

•Development of isogenic RKO cell model systems to analyze the effect of wild-type or phospho-mutant Bcl2 expression on mitotic events and susceptibility to Taxol-mediated apoptosis.

•Development of a 2D isoelectric focusing assay to analyze Bcl2 phosphorylation status in protein lysates prepared from tumor specimens.

• Our results indicate that expression of WT or S70A Bcl-2 did not affect sensitivity of established xenograft tumors to Taxol.

•Increased expression of WT Bcl-2, but not S70A Bcl-2, could promote tumor cell survival and proliferation in an anchorage independent manner after transient Taxol exposure.

REPORTABLE OUTCOMES

•Stewart, Z.A., Tang, L.J., and Pietenpol, J.A. (2001) Increased p53 phosphorylation after microtubule disruption is mediated in a microtubule inhibitor- and cell-specific manner. Oncogene 20, 113-124.

•Stewart, Z.A. And Pietenpol, J.A. (2001) "G2 Checkpoint and Anticancer Therapy," in Cell Cycle. Edited by Mikhail Blagosklonny. Eurekah Publishers, Georgetown.

•Scatena, C.D., Tang, L.J., and Pietenpol, J.A. Ectopic Expression of Wild-Type Bcl-2, not S70A Phosphomutant Bcl-2, Confers Increased Cell Survival after Transient Taxol Treatment. Submitted.

CONCLUSIONS

The main objective of this research is to test the hypothesis that prolonged activation of cdc2 activation in mitosis and subsequent phosphorylation of Bcl2 are required for Taxol-mediated apoptosis in breast cancer cells. The ultimate goal of our research is to identify a signaling cascade that is important for the cytotoxic effects of Taxol and determine if this signaling cascade is operational *in vivo* in breast carcinomas treated with Taxol. Our progress described above continues to forge the the link between Taxol modulation of microtubule dynamics, mitotic block, and cell death and promises to lead to improved clinical use of the drug in the treatment of breast cancer. It is critical to understand how Taxol works at the molecular level in order to pursue rational design of new drugs for the treatment of breast cancer that act through alteration of mitotic signaling pathways.

Furthermore, these studies continue to represent a novel area of investigation — exploring cell cycle changes *in vitro* and *in vivo* after Taxol treatment of breast cancer tumor cells. In fact, the data from the first first year of funding provided the necessary preliminary data to proceed with similar studies using tumor material from patients enrolled in a Taxol-based clinical trial at the Vanderbilt-Ingram Cancer Center (these related studies were funded by a Discovery grant from the Vanderbilt-Ingram Cancer Center). Our results from analyses of human tumors parallels those we have generated with xenograft tumors in mice and is now being used as the basis for a project we are preparing for theVanderbilt-Ingram Cancer Center Breast SPORE grant application that will be submitted to the NCI, February 2001.

APPENDIX

Contents: 2 publication and a submitted manuscript

•Stewart, Z.A., Tang, L.J., and Pietenpol, J.A. (2001) Increased p53 phosphorylation after microtubule disruption is mediated in a microtubule inhibitor- and cell-specific manner. Oncogene 20, 113-124.

•Stewart, Z.A. And Pietenpol, J.A. (2001) "G2 Checkpoint and Anticancer Therapy," in Cell Cycle. Edited by Mikhail Blagosklonny. Eurekah Publishers, Georgetown.

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Oncogene (2001) 20, 113–124

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Increased p53 phosphorylation after microtubule disruption is mediated in a microtubule inhibitor- and cell-specific manner

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p53 is present at low levels in unstressed cells. Numerous cellular insults, including DNA damage and microtubule disruption, elevate p53 protein levels. Phosphorylation of p53 is proposed to be important for p53 stabilization and activation after genotoxic stress; however, p53 phosphorylation after microtubule disruption has not been analysed. The goal of the current study was to determine if p53 phosphorylation increases after microtubule disruption, and if so, to identify specific p53 residues necessary for microtubule inhibitor-induced phosphorylation. Two dimensional gel analyses demonstrated that the number of p53 phospho-forms in cells increased after treatment with microtubule inhibitors (MTIs) and that the pattern of p53 phosphorylation was distinct from that observed after DNA damage. p53 phosphorylation also varied in a MTI-dependent manner, as Taxol and Vincristine induced more p53 phospho-forms than nocodazole. Further, MTI treatment increased phosphorylation of p53 on serine-15 in epithelial tumor cells. In contrast, serine-15 phosphorylation of p53 did not increase in MTI-treated primary cultures of human fibroblasts. Analysis of ectopically expressed p53 phospho-mutant proteins from Taxol- and nocodazole-treated cells indicated that multiple p53 amino terminal residues, including serine-15 and threonine-18, were required for Taxol-mediated phosphorylation of p53. Taken together, the results of this study demonstrate that distinct p53 phospho-forms are induced by MTI treatment as compared to DNA damage and that p53 phosphorylation is mediated in a MTI- and cell-specific manner. *Oncogene* (2001) **20,** 113–124.

Keywords: taxol; vincristine; nocodazole; adriamycin; ionizing radiation; ATM

Introduction

The p53 tumor suppressor protein is present at low levels in unstressed cells, likely due to MDM2 binding and targeting p53 for ubiquitin-mediated degradation (Kubbutat *et al.*, 1997; Haupt *et al.*, 1997). However, cellular insults, such as DNA damage, oncogenic stimuli, or microtubule disruption, elevate p53 protein levels. While upstream p53 signaling pathways induced by genotoxic stress and oncogenic stimuli have been identified, pathways that mediate p53 activation after microtubule disruption have not been elucidated. Cells lacking p53-dependent signaling undergo endoreduplication and become polyploid after microtubule inhibitor (MTI) treatment (Cross *et al.*, 1995; Di Leonardo *et al.*, 1997; Khan and Wahl, 1998; Lanni and Jacks, 1998; Stewart *et al.*, 1999a). Further, tumor cells with defective p53-mediated G1/S checkpoint function are sensitized to MTI treatment *in vivo* (Stewart *et al.*, 1999b). Since drugs that alter microtubule dynamics are widely used chemotherapeutic agents, identifying the signaling pathways involved in p53 activation after microtubule disruption may have important therapeutic implications.

After genotoxic stress, phosphorylation of p53 may be important for p53 stabilization. Numerous cellular kinases mediate phosphorylation of p53 after ionizing radiation (IR) and ultraviolet radiation (UV). Several members of the phosphoinositide-3 kinase (PI-3K) family can directly phosphorylate p53 on amino terminal residues after both IR and UV treatment, including DNA-activated protein kinase (DNA-PK), ataxia-telangiectasia mutated (ATM) kinase and ATM-related kinase (ATR) (Lees-Miller *et al.*, 1992; Canman *et al.*, 1998; Banin *et al.*, 1998; Tibbetts *et al.*, 1999; Hall-Jackson *et al.*, 1999). DNA-PK is activated by DNA double-strand breaks and phosphorylates p53 on Ser-15 and Ser-37 *in vitro* (Lees-Miller *et al.*, 1992). ATM is also activated by DNA double-strand breaks and phosphorylates p53 on Ser-15 *in vitro* (Canman and Lim, 1998; Canman *et al.*, 1998). ATM function is defective in patients with ataxia-telangiectasia (AT), a disorder in which patients have increased sensitivity to radiation and are highly cancer prone (Lavin and Shiloh, 1996). Cells from AT patients have delayed p53 stabilization and significantly diminished Ser-15 phosphorylation after IR exposure (Siliciano *et al.*, 1997; Shieh *et al.*, 1997; Canman *et al.*, 1998). ATR phosphorylates p53 on both Ser-15 and Ser-37 *in vitro* and overexpression of a catalytically inactive ATR reduces Ser-15 phosphorylation at late times after IR (Tibbetts *et al.*, 1999; Hall-Jackson *et al.*, 1999). DNA-PK and ATM also phosphorylate MDM2 after DNA damage, preventing formation of the p53-MDM2 complex (Mayo *et al.*, 1997; Khosravi *et al.*, 1999).

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Thus, post-translational regulation of the p53-MDM2 interaction likely occurs by modifications on both proteins.

ATM-dependent signaling induced by DNA damage also results in the activation of the Chk1 and Chk2 kinases (Matsuoka *et al.*, 1998; Sanchez *et al.*, 1997; Furnari *et al.*, 1997). Chk1- and Chk2-mediated phosphorylation of p53 on Ser-20 may be important for p53 stabilization after DNA damage (Hirao *et al.*, 2000; Shieh *et al.*, 2000; Chehab *et al.*, 2000). Chk2 phosphorylates p53 on Ser-20 *in vitro* and this phosphorylation dissociates p53-MDM2 complexes (Chehab *et al.*, 2000). Additionally, Chk2−/− embryonic stem cells fail to maintain IR-induced cell cycle arrest, a phenotype similar to that of p53−/− cells (Hirao *et al.*, 2000). Further, wild type (wt) Chk1 phosphorylates p53 on Ser-20 *in vitro* and overexpression of Chk1 increases levels of p53 protein in cells (Shieh *et al.*, 2000). Since mutation of Ser-20 to alanine abrogates p53 stabilization after both IR and UV exposure (Chehab *et al.*, 1999), Chk1 and Chk2 may play an important role in DNA damage-induced p53 activation.

Several other cellular kinases mediate phosphorylation of p53 after genotoxic stress. The p38 stress-activated kinase phosphorylates p53 on Ser-33 and Ser-46 afer UV radiation but not IR (Bulavin *et al.*, 1999). Inhibition of p38 kinase activity or mutation of Ser-33 and Ser-46 to alanines reduced p53-mediated apoptosis and decreased sensitivity to UV radiation, suggesting p38 plays an important role in p53 signaling after UV exposure (Bulavin *et al.*, 1999). Casein kinase I (CKI) phosphorylates murine p53 on Ser-4, Ser-6, and Ser-9 *in vitro* (Milne *et al.*, 1992; Knippschild *et al.*, 1997). CKI also phosphorylates human p53 on Thr-18 *in vitro*, a modification that required prior Ser-15 phosphorylation (Dumaz *et al.*, 1999; Sakaguchi *et al.*, 2000). However, further studies are necessary to determine if CKI phosphorylates p53 on Thr-18 after DNA damage *in vivo*.

Numerous kinases and phosphatases regulate the phosphorylation of p53 on carboxy terminal sites. Both casein kinase II and the double stranded RNA-activated protein kinase PKR phosphorylate p53 on Ser-392, a modification proposed to control p53-mediated transactivation (Hupp *et al.*, 1992; Hall *et al.*, 1996; Cuddihy *et al.*, 1999). Protein kinase C mediates phosphorylation of three carboxy terminal serine residues of p53: Ser-371, Ser-376, and Ser-378 (Baudier *et al.*, 1992). Of note, Ser-376 and Ser-378 are constitutively phosphorylated in cells, and Ser-376 is dephosphorylated in response to DNA damage in an ATM-dependent manner (Waterman *et al.*, 1998). Dephosphorylation of Ser-376 results in enhanced binding of 14-3-3 proteins to p53 and mediates increased sequence-specific DNA binding by p53 (Waterman *et al.*, 1998). Finally, phosphorylation of Ser-315 by Cdk2 and Cdc2, as well as dephosphorylation by Cdc14, may influence the nuclear localization of p53 and regulate p53 binding to a subset of p5t3 consensus DNA binding sites (Wang and Prives, 1995; Hecker *et al.*, 1996; Li *et al.*, 2000).

Several p53 upstream signaling pathways induced by genotoxic stress and oncogenic stimuli (Sherr and Weber, 2000) have been determined in recent years; however, the mechanism(s) by which p53 is activated after microtubule disruption has not been identified. The goal of the current study was to determine if p53 phosphorylation increases after microtubule disruption, and if so, to identify specific p53 residues necessary for MTI-induced phosphorylation. The results demonstrate that distinct p53 phospho-forms are induced by MTI treatment as compared to DNA damage. Phosphorylation of p53 on Ser-15 increased in epithelial tumor cells but not in non-transformed fibroblasts after microtubule disruption. Further, multiple p53 amino terminal residues, including Ser-15 and Thr-18, were required for Taxol-induced phosphorylation of ectopically expressed p53 phospho-mutant proteins.

Results

Taxol and vincristine treatments induce multiple p53 phospho-forms

To analyse p53 phosphorylation after microtubule disruption, protein lysates were prepared from control and Taxol-treated HCT116 cells, and p53 was analysed by two dimensional (2D) gel electrophoresis. Taxol treatment of HCT116 cells resulted in a significant increase in the number and amount of phospho-forms detected by 2D analysis as compared to those observed in a control lysate (Figure 1a). We analysed 1 mg of control lysates as compared to 150 μg of protein lysate from Taxol-treated cells to ensure that the additional p53 phospho-forms observed in the treated samples were a reflection of stress-induced changes and not merely higher p53 protein levels. The faster migrating phospho-form labeled S represents an internal standard provided by the admixture of a recombinant carboxy terminal truncated human p53 protein (amino acids 1–353) (Szak and Pietenpol, 1999) to the IEF sample buffer (Figure 1a). To confirm that the multiple p53 species detected by 2D analysis were a result of phosphorylation, an aliquot of the Taxol-treated HCT116 lysate was incubated with potato acid phosphatase prior to 2D analysis. Phosphatase treatment collapsed the phospho-forms to a single species of protein labeled P_0 (Figure 1a). Sequential phospho-forms are indicated as P_1 and P_2; phospho-forms beyond P_2 were not assigned. Of note, each of the various p53 phospho-forms (P_0, P_1, P_2, etc.) likely comprise a mixture of phosphorylated p53 species with identical mass/charge ratios.

To compare MTI-induced p53 phosphorylation to that induced by genotoxic stress, protein lysates were prepared from HCT116 cells treated with MTIs (Taxol, Vincristine, nocodazole) and DNA damaging agents (IR, ADR, UV) and evaluated by 2D gel electrophoresis. All treatments except nocodazole induced an increase in the relative number of p53 phospho-forms beyond those present in the control lysate, as well as a

distinct p53 phosphorylation pattern (Figure 1b). The different patterns of p53 phospho-forms induced by Taxol, Vincristine, and nocodazole treatments also suggest distinct signaling pathways are activated by these MTIs (Figure 1b).

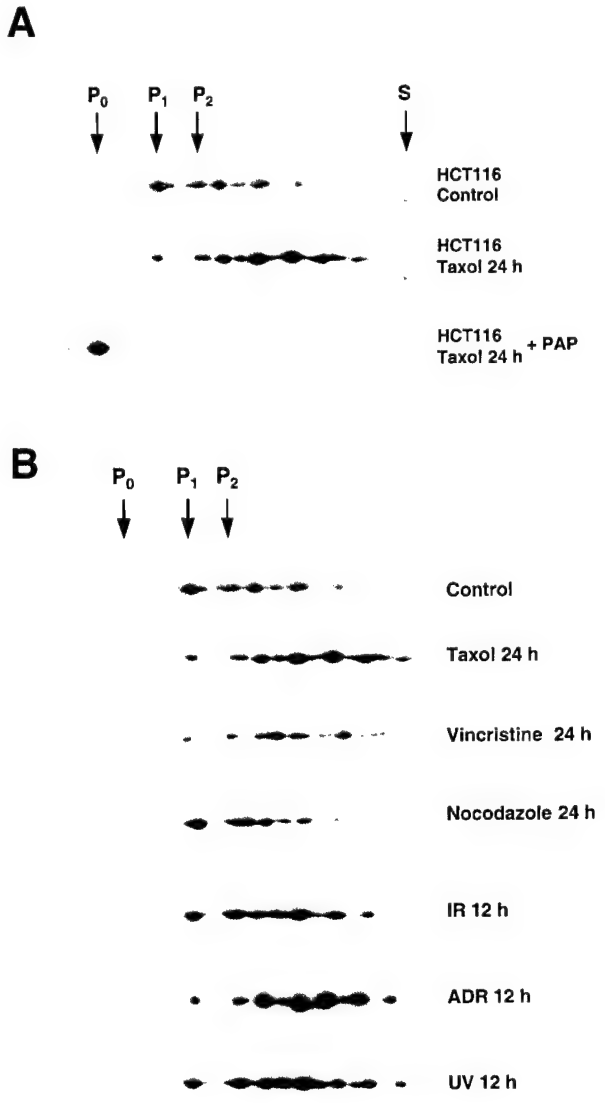


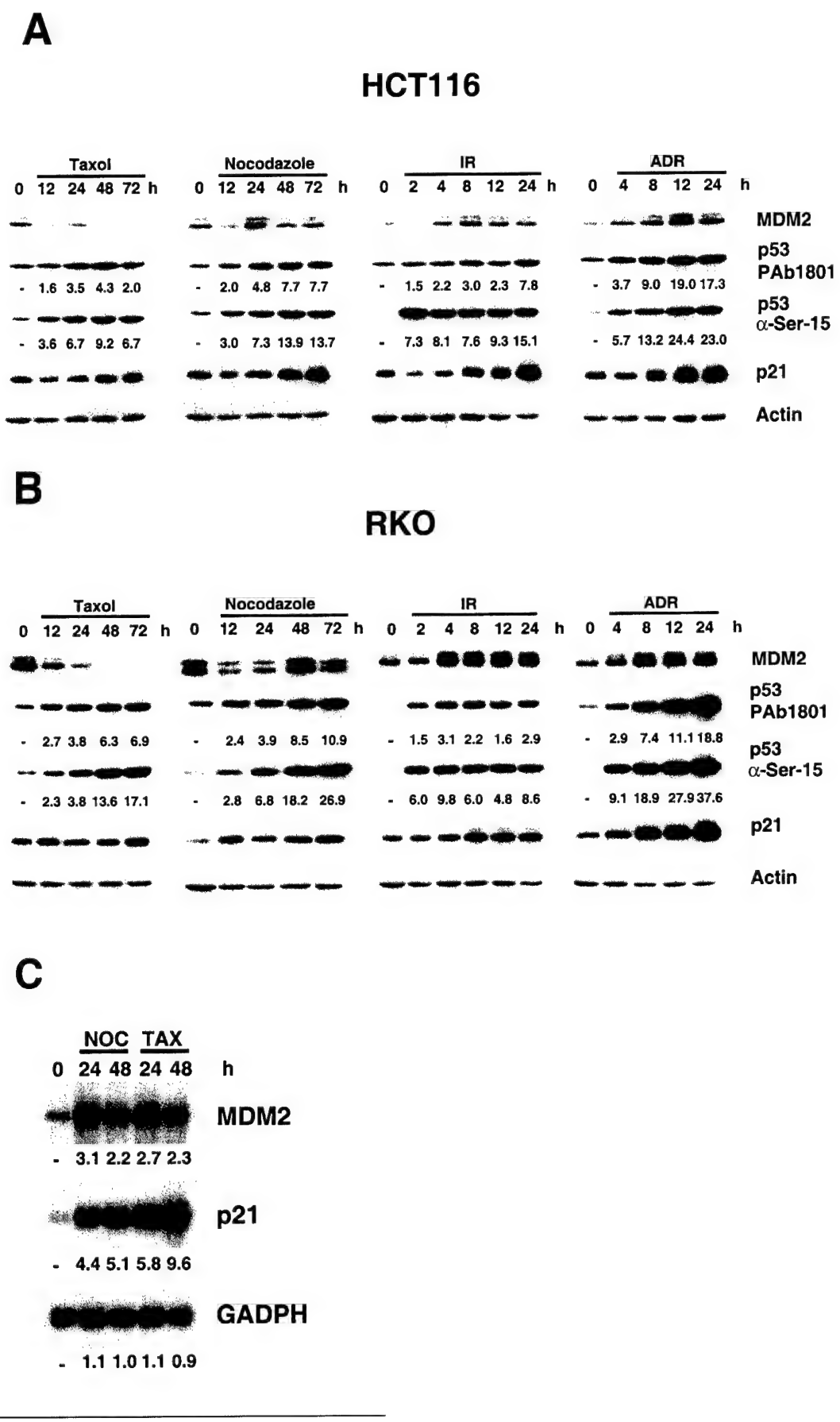
Figure 1 Taxol and Vincristine treatments induce multiple p53 phospho-forms. (**a**) Lysates from control and Taxol-treated (100 nM) HCT116 cells were analysed by 2D gel electrophoresis. To verify that the p53 forms were a result of phosphorylation, parallel lysates prepared from Taxol-treated cells were treated with potato acid phosphatase (PAP) prior to 2D gel analysis. S denotes the migration of the internal standard protein, a truncated form of human p53. P_0 indicates the migration of unphosphorylated p53, while P_1 and P_2 denote various phospho-forms of p53. The various p53 phospho-forms (P_0, P_1, P_2) likely comprise a mixture of phosphorylated p53 species with identical mass/charge ratios. (**b**) Lysates from control and cells treated with Taxol (100 nM), Vincristine (100 nM), nocodazole (83 nM), IR (10 Gy), ADR (350 nM), or UV (100 mJ) for the indicated times were analysed by 2D gel electrophoresis. Samples were evaluated with the PAb1801 antibody. Results are representative of three independent experiments

Serine-15 phosphorylation of p53 is increased in MTI-treated tumor cells

After exposure of cells to genotoxic agents, p53 phosphorylation on Ser-15 increases and is proposed to play a role in p53 stabilization (Canman *et al*., 1998; Banin *et al*., 1998; Craig *et al*., 1999). To determine if microtubule disruption induces phosphorylation of p53 on Ser-15, protein lysates from HCT116 cells treated with Taxol and nocodazole were examined by Western analysis using an antibody specific to p53 phosphorylated at Ser-15. Samples were also evaluated to assess total p53 protein levels and changes in the protein levels of the p53 downstream targets MDM2 and p21. For comparison, lysates from IR- and ADR-treated HCT116 cells were also analysed.

After treatment of HCT116 cells with Taxol or nocodazole, an increase in total p53 protein levels was detectable by 12 h (Figure 2a, PAb1801). The increase in p53 after nocodazole treatment was accompanied by elevations in MDM2 and p21 protein levels. In contrast, after Taxol treatment, the elevation in p53 protein was accompanied by a decrease in MDM2 protein levels (Figure 2a). Ser-15 phosphorylation of p53 also increased above control levels in nocodazole- and Taxol-treated cells. Fluoroimager analyses were performed to quantify the PAb1801 and the α-Ser-15 p53 chemiluminescent signals to determine the fold increase of total p53 and Ser-15 phosphorylated p53 relative to control for each treatment; these numbers appear below the PAb1801 and the α-Ser-15 p53 panels in Figure 2a. Treatment with both MTIs increased p53 Ser-15 phosphorylation for all timepoints evaluated (Figure 2a). For example, after 12 h Taxol treatment, there was a 3.6-fold increase in Ser-15 phosphorylated p53 as compared to a 1.6-fold increase in total p53 protein (Figure 2a). After IR and ADR treatments, the levels of total p53 protein and Ser-15 phosphorylated p53 increased for all timepoints examined, consistent with previous studies (Canman *et al*., 1998; Gao *et al*., 1999), and was accompanied by elevations in MDM2 and p21 protein levels (Figure 2a).

To verify the results obtained in the HCT116 cells, total p53 and Ser-15 phosphorylated p53 levels were analysed in the RKO human colon carcinoma cell line after treatment (Figure 2b). Similar to HCT116 cells, MTIs and DNA damaging agents induced an elevation in Ser-15 phosphorylated p53 greater than the elevation in total p53 protein for all timepoints (Figure 2b). For example, after 48 h nocodazole treatment, there was a 18.2-fold increase in Ser-15 phosphorylated p53 as compared to a 8.5-fold increase in total p53 protein (Figure 2b). Comparable increases in Ser-15 phosphorylated p53 and total p53 were observed after 48 h Taxol treatment, as the increase in Ser-15 phosphorylated p53 was a fold greater than that observed for total p53 protein levels (Figure 2b). Like the HCT116 cells, the increase in p53 after nocodazole, IR, and ADR treatments was accompanied by elevations in


A
HCT116
Taxol
0 12 24 48 72 h
Nocodazole
0 12 24 48 72 h
IR
0 2 4 8 12 24 h
ADR
0 4 8 12 24 h
MDM2
p53 PAb1801
- 1.6 3.5 4.3 2.0
- 2.0 4.8 7.7 7.7
- 1.5 2.2 3.0 2.3 7.8
- 3.7 9.0 19.0 17.3
p53 α-Ser-15
- 3.6 6.7 9.2 6.7
- 3.0 7.3 13.9 13.7
- 7.3 8.1 7.6 9.3 15.1
- 5.7 13.2 24.4 23.0
p21
Actin
B
RKO
Taxol
0 12 24 48 72 h
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- 2.7 3.8 6.3 6.9
- 2.4 3.9 8.5 10.9
- 1.5 3.1 2.2 1.6 2.9
- 2.9 7.4 11.1 18.8
p53 α-Ser-15
- 2.3 3.8 13.6 17.1
- 2.8 6.8 18.2 26.9
- 6.0 9.8 6.0 4.8 8.6
- 9.1 18.9 27.9 37.6
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MDM2 and p21 protein levels, while MDM2 protein levels decreased after Taxol treatment in the RKO cells (Figure 2b).

To determine if the decrease in MDM2 protein levels after Taxol treatment was mediated by transcriptional regulation of the MDM2 promoter, Northern analyses were performed on mRNA isolated from nocodazole- and Taxol-treated HCT116 cells. Both nocodazole and Taxol treatment resulted in increased MDM2 and p21 mRNA levels after 24 and 48 h of treatment (Figure 2c). Thus, the reduction in MDM2 protein levels after Taxol treatment was not a consequence of a decrease in MDM2 mRNA.

Increased serine-15 phosphorylation of p53 after MTI treatment occurs in a cell-specific manner

ATM is required for p53 stabilization and Ser-15 phosphorylation after IR, as cells from AT patients have significantly reduced p53 protein levels and Ser-15 phosphorylation as compared to normal cells after IR (Siliciano *et al*., 1997; Shieh *et al*., 1997; Canman *et al*., 1998; Banin *et al*., 1998). To determine if ATM plays a role in MTI-induced elevation of Ser-15 phosphorylation, ATM-deficient fibroblasts (AT-1) were treated with Taxol or nocodazole and the relative levels of Ser-15 phosphorylated p53 determined by Western. MTI treatment did not increase Ser-15 phosphorylation of p53 in the AT-1 fibroblasts after adjustment for the increase in total p53 protein (Figure 3a). However, p53 was not significantly phosphorylated on Ser-15 after MTI treatment in normal human dermal fibroblasts (NHDFs) (Figure 3b), suggesting MTIs induce distinct signaling pathways in fibroblasts that do not increase Ser-15 phosphorylation. Of note, the MTI-mediated increases in total p53 were not significantly reduced in AT-1 cells as compared to the fold increases observed in NHDFs, suggesting that MTI-induced stabilization of p53 is not ATM-dependent (Figure 3). Further, similar p53 phospho-forms were observed in lysates prepared from Taxol-treated NHDF and AT-1 fibroblasts (data not shown).

NHDFs and AT-1 cells were also treated with IR and ADR to verify that Ser-15 phosphorylation of p53 could occur in NHDFs (Figure 3). IR and ADR treatments increased Ser-15 phosphorylated p53 in both NHDFs and AT-1 cells; however, the IR and ADR-induced elevations in total p53 protein levels and Ser-15 phosphorylated p53 were greater in NHDFs as compared to AT-1 fibroblasts (Figure 3).

Analysis of p53 amino terminus phospho-mutant proteins after treatment of cells with MTIs, IR, and ADR

To further investigate p53 phosphorylation after microtubule disruption, p53-deficient H1299 cells were transfected with a panel of expression vectors encoding phosphorylation-site mutant p53 proteins, the cells were treated with Taxol or nocodazole, and the ectopically expressed p53 proteins were examined by 2D gel electrophoresis. The proteins analysed in this study include the following point mutants in which serine or threonine were mutated to alanine: Ser-15 (S15A), Thr-18 (T18A), Ser-20 (S20A), Ser-37 (S37A) (Ashcroft *et al*., 1999). A Thr-18 point mutant in which threonine was mutated to aspartic acid (T18D) (Ashcroft *et al*., 1999) and a double mutant in which Ser-15 and Thr-18 (S15A/T18A) (Blattner *et al*., 1999) were altered to alanine were also used in these experiments. In addition, a p53 mutant (N/C-Term) in which a majority of the previously described p53 amino terminal and carboxy terminal phosphorylation sites were mutated to alanine was examined; this protein contained alanine substitutions at residues 6, 9, 15, 18, 20, 33, 37, 315, 371, 376, 378 and 392 (Ashcroft *et al*., 1999). Expression of each p53 mutant protein was verified by Western (data not shown).

To determine if ectopically expressed wt p53 was phosphorylated after Taxol treatment, wt p53 was transiently expressed in H1299 cells in the presence or absence of Taxol and evaluated by 2D analysis. A similar number of wt p53 phospho-forms were observed in Taxol-treated H1299 cells as compared to endogenous p53 in Taxol-treated HCT116 cells (Figures 1a and 4a). Further, p53 protein isolated from Taxol-treated H1299 cells had significantly more phospho-forms than the untreated wt p53 control, verifying that ectopically expressed p53 was phosphorylated after MTI treatment (Figure 4a). Alignment by the internal standard protein allowed assignment of P_1, P_2, and P_3 for the ectopically expressed p53 proteins (Figure 4a, S). Initial 2D analysis of each mutant protein was performed in the absence of cellular stress. All the mutants, with the exception of T18D, had a reduced number of p53 phospho-forms as compared to wt p53 (Figure 4b). A significant fraction of the T18A mutant protein was present as the P_1 phospho-form as compared to wt p53 or any other point mutant (Figure 4b). In addition, the N/C-Term p53 mutant migrated at predominantly the P_0 form, a finding consistent with the removal of the majority of

Figure 2 Increased phosphorylation of p53 on Ser-15 in tumor cells after MTI treatment. Western analysis of MDM2, total p53 (PAb1801), Ser-15 phosphorylated p53 (α-Ser-15), p21, and actin protein levels in HCT116 (**a**) or RKO (**b**) cells after treatment with Taxol (100 nM), nocodazole (83 nM), IR (10 Gy), and ADR (350 nM). The numbers below the total p53 and Ser-15 phosphorylated p53 panels represent fold increase relative to the 0 h timepoint. (**c**) Northern analysis of MDM2, p21 and GADPH mRNA levels in HCT116 cells after treatment with nocodazole (83 nM) or Taxol (100 nM). The numbers below each panel represent the fold increase relative to the 0 h timepoint. Results are representative of two independent experiments

the previously described p53 phosphorylation sites (Figure 4b).

To determine if mutation of specific phospho-residues affected the MTI-induced phosphorylation of p53, each of the mutant proteins was expressed in H1299 cells, the cells were treated with Taxol or nocodazole for 24 h, and the ectopically expressed p53 proteins were examined by 2D analysis. Taxol treatment did not significantly increase phosphorylation of the N/C-Term mutant, as the majority of the protein remained as the P_0 form, suggesting Taxol-induced phosphorylation was dependent on the mutated p53 phospho-residues (Figure 5a). All of the amino terminal point mutants examined had reduced p53 phosphorylation as compared to wt p53 after Taxol treatment (Figure 5a). For example, Taxol

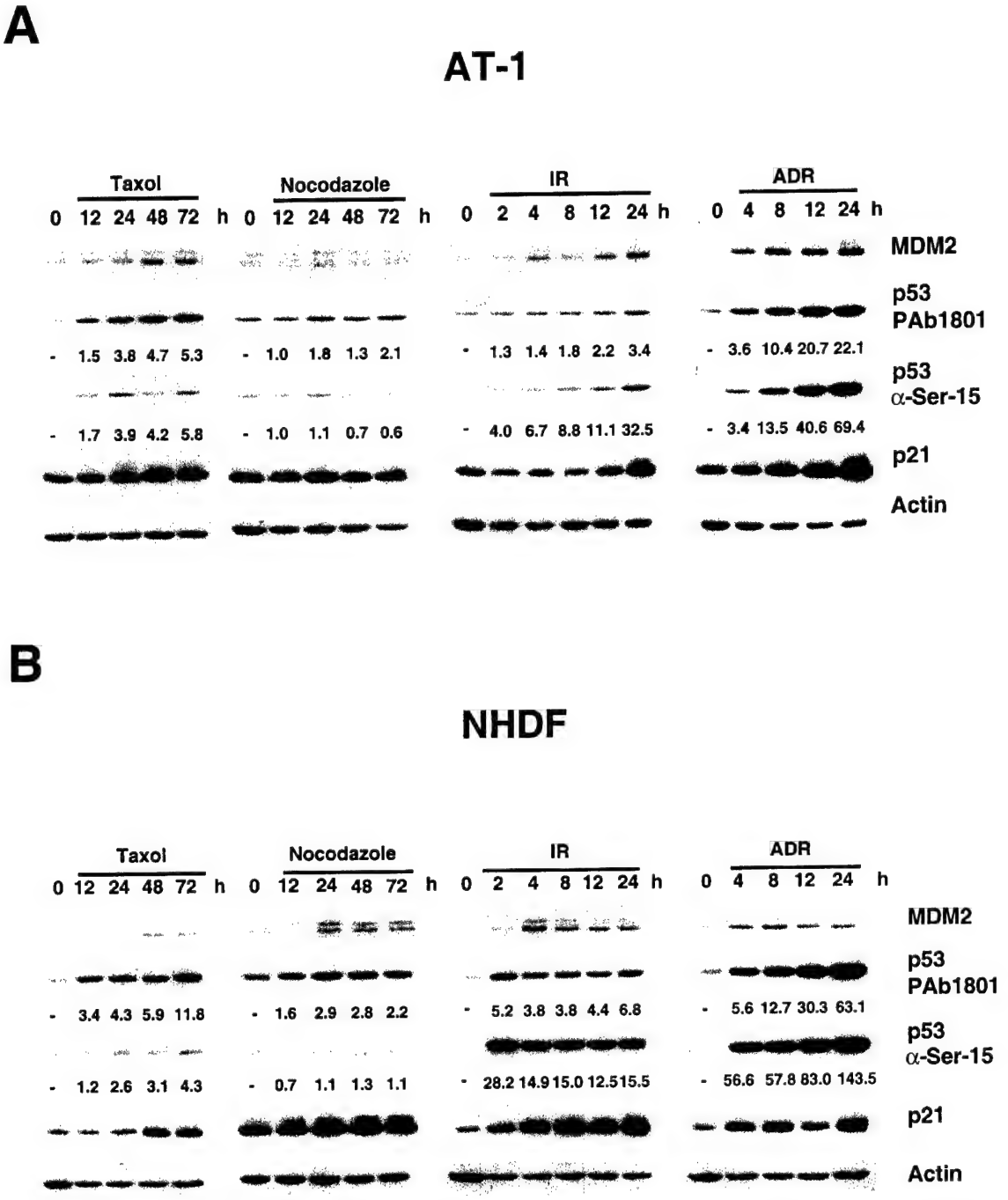

Figure 3 ATM-deficient cells have a delayed increase in p53 protein levels after Taxol treatment. Western analysis of MDM2, total p53 (PAb1801), Ser-15 phosphorylated p53 (α-Ser-15), p21, and actin protein levels in AT-1 (**a**) or NHDF (**b**) cells after treatment with Taxol (100 nM), nocodazole (83 nM), IR (10 Gy), and ADR (350 nM). Results are representative of three independent experiments

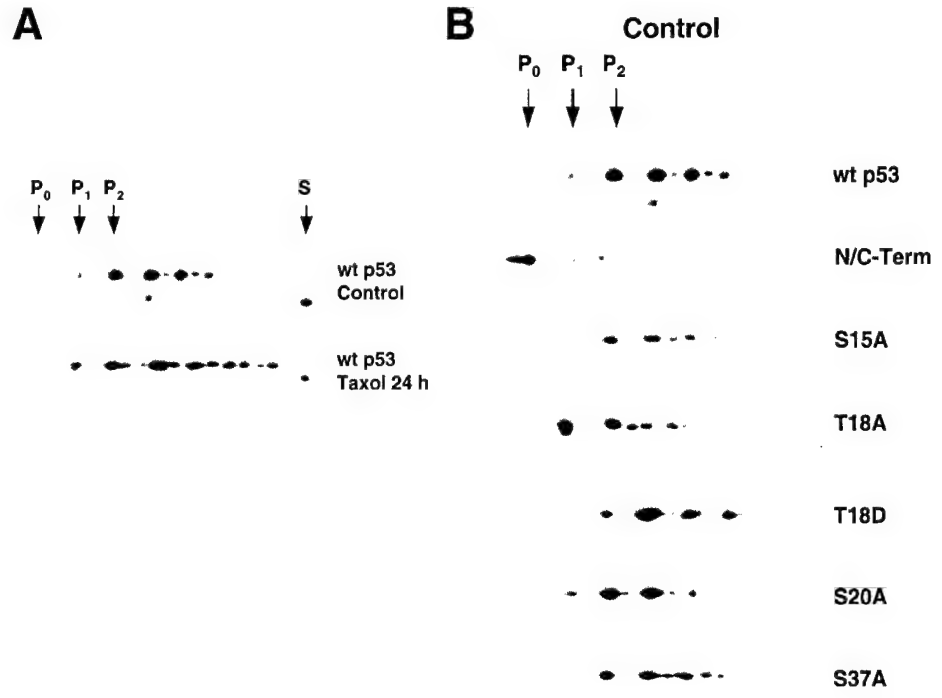

Figure 4 Taxol treatment induces phosphorylation of ectopically expressed p53 on multiple sites. (**a**) Lysates from control and Taxol-treated (100 nM) H1299 cells transfected with wt p53 were analysed by 2D gel electrophoresis. The P_0 and the P_1 and P_2 phospho-forms in the ectopically expressed p53 proteins were assigned by alignment with the internal standard protein (S). (**b**) Lysates from untreated H1299 cells transfected with wt p53 or the indicated p53 point mutants were analysed by 2D gel electrophoresis. P_0 and the P_1 and P_2 phospho-forms were assigned by alignment with the internal standard protein. The various p53 phospho-forms (P_0, P_1, P_2) likely comprise a mixture of phosphorylated p53 species with identical mass/charge ratios. Results are representative of three independent experiments

treatment did not increase the number of S15A mutant phospho-forms beyond those observed in the control wt p53. This result was consistent with the detection of Ser-15 phosphorylation in HCT116 and RKO cells after Taxol treatment (Figure 2a,b). The T18A mutant had the most significant reduction in phosphorylation of all the p53 single point mutants evaluated with the majority of the protein in the P_1 form and a lesser amount at P_2 (Figure 5a). Further, replacement of Thr-18 with aspartic acid (T18D) partially rescued p53 phosphorylation (Figure 5a). Taken together, these data suggest that Taxol-induced phosphorylation of p53 requires multiple amino terminal residues that function either as direct phosphorylation sites or as recognition sequences for kinases that phosphorylate other p53 residues.

Similar to Taxol treatment, the nocodazole-treated N/C-Term mutant remained predominantly in the P_1 form (Figure 5b). In contrast to Taxol treatment, mutation of individual p53 amino terminal residues did not significantly impair nocodazole-mediated phosphorylation, suggesting that distinct signaling pathways are activated by these two MTIs (Figure 5b). For example, nocodazole-mediated phosphorylation of the T18A mutant protein resembled that observed with wt p53, with the majority of the protein in the P_2 or greater phosphorylated forms. This result is significantly different from that observed after Taxol treatment, as the majority of the T18A protein was in the P_1 phospho-form after Taxol exposure (Figure 5a,b). Thus, similar to the 2D data obtained with endogenous p53 in HCT116 cells (Figure 1b), analysis of ectopically expressed p53 point mutant proteins suggests that Taxol and nocodazole induce distinct p53 phosphorylation patterns.

Analysis of IR- and ADR-induced phosphorylation of the p53 mutant proteins demonstrated that the S37A mutant had the most significant reduction in p53 phosphorylation as compared to wt p53, suggesting that phosphorylation of Ser-37 is important for subsequent phosphorylation of p53 after genotoxic stress (Figure 5c,d). Like Taxol and nocodazole treatment, IR and ADR treatment did not significantly increase the p53 phospho-forms detected in the N/C-Term mutant (Figure 5c,d). After IR or ADR exposure, the majority of the T18A mutant was present as P_2 or greater phospho-forms (Figure 5c,d), in contrast to Taxol treatment, in which a significant fraction of the T18A mutant protein was present as the P_1 phospho-form (Figure 5a).

To further investigate p53 phosphorylation after MTI treatment, the S15A/T18A double mutant was analysed by 2D electrophoresis. Similar to the untreated T18A mutant, the control S15A/T18A mutant protein had a significantly higher level of the P_1 phospho-form as compared to wt p53 or any other

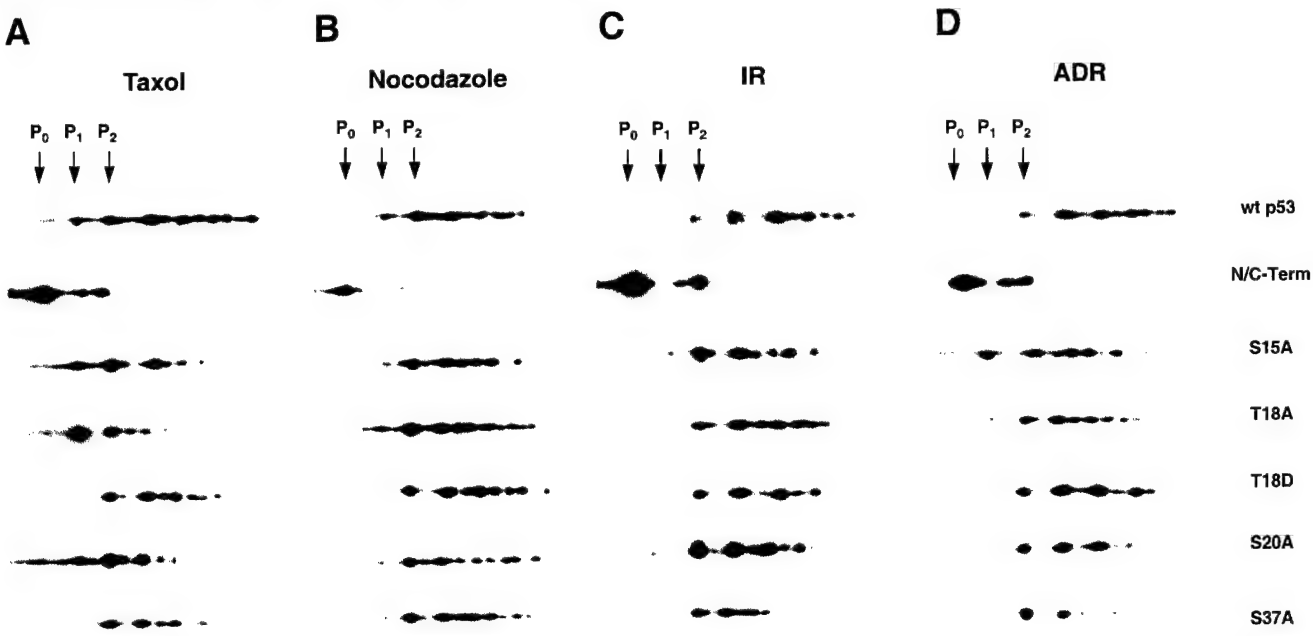

Figure 5 Differential phosphorylation of p53 amino terminal point mutant proteins after MTI, IR, or ADR treatment. Lysates from H1299 cells transfected with wt p53 or the indicated p53 point mutants and treated with (**a**) Taxol (100 nM), (**b**) nocodazole (83 nM), (**c**) IR (10 Gy), or (**d**) ADR (350 nM) were analysed by 2D gel electrophoresis. P_0 and the P_1 and P_2 phospho-forms were assigned by alignment with the internal standard protein. The various p53 phospho-forms (P_0, P_1, P_2) likely comprise a mixture of phosphorylated p53 species with identical mass/charge ratios. Results are representative of two independent experiments

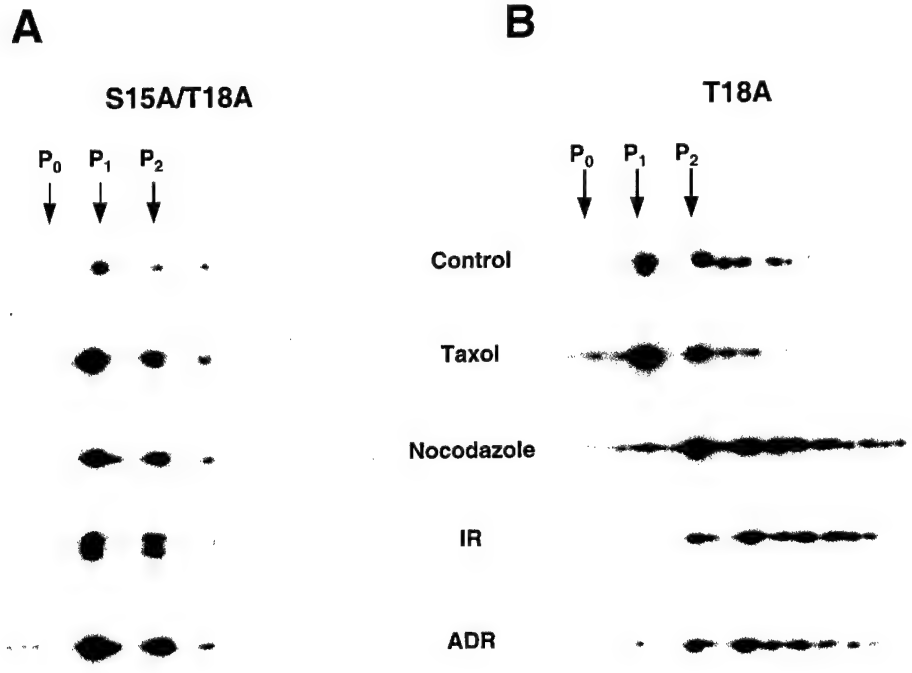

Figure 6 Mutation of Ser-15 and Thr-18 results in reduced phosphorylation of p53 after treatment with MTIs and genotoxic agents. Lysates from H1299 cells transfected with either the S15A/T18A p53 double point mutant (**a**) or the T18A p53 point mutant (**b**) and treated with Taxol (100 nM), nocodazole (83 nM), IR (10 Gy), and ADR (350 nM) were analysed by 2D gel electrophoresis. The various p53 phospho-forms (P_0, P_1, P_2) likely comprise a mixture of phosphorylated p53 species with identical mass/charge ratios. Results are representative of two independent experiments

individual point mutant protein (Figures 4b and 6a). In contrast to the single point mutant proteins evaluated, the S15A/T18A mutant protein had a significant reduction in phosphorylation after treatment with either MTIs or genotoxic agents, as the majority of the protein migrated as the P_1 and P_2 phospho-forms (Figure 6a). This finding differs from the phosphorylation patterns observed with either the S15A or T18A point mutant proteins. For example, the S15A mutant protein had reduced phosphorylation after Taxol, nocodazole, IR and ADR treatment as compared to wt p53, but all treatments induced phosphorylation beyond the P_2 phospho-form (Figure 5). Similarly, while the T18A mutant protein had a reduction in phospho-forms after Taxol treatment, it was phosphorylated beyond the P_2 phospho-form after nocodazole, IR, or ADR treatment (Figure 6b). These results suggest that under all conditions examined, phosphorylation of Ser-15 or Thr-18 is required for phosphorylation of subsequent sites.

Discussion

Recent studies have identified signaling pathways that regulate p53 activation after DNA damage or oncogene activation; however, the mechanism(s) by which p53 is activated after microtubule disruption has not been elucidated. The goal of the current study was to analyse p53 phosphorylation after microtubule disruption and to identify specific p53 residues necessary for MTI-induced phosphorylation. Two dimensional gel electrophoresis of p53 from MTI-treated HCT116 cells demonstrated that p53 phosphorylation increased after microtubule disruption and that Vincristine and Taxol induced significantly more phospho-forms than nocodazole. Further, mutation of p53 amino terminal phosphorylation sites significantly impaired Taxol-induced phosphorylation of p53, while nocodazole-mediated phosphorylation was minimally reduced. These data suggest that different upstream signaling pathways are activated by specific MTIs and result in distinct phosphorylation patterns of p53. Further, 2D analysis of ectopically expressed p53 phospho-mutant proteins demonstrated that Thr-18 is a critical residue for subsequent phosphorylation of p53 after Taxol treatment, while Ser-37 is an important residue for subsequent p53 phosphorylation after genotoxic stress. It remains to be determined if Thr-18 and Ser-37 function as direct phosphorylation sites or as part of recognition sequences for kinases that phosphorylate other p53 residues.

In HCT116 and RKO cells, MDM2 mRNA and protein levels increased after nocodazole treatment, whereas after Taxol treatment, MDM2 protein levels decreased, despite an increase in MDM2 mRNA levels. In contrast, both MTIs induced an elevation in p21 mRNA and protein levels. Several groups have reported differential upregulation of p21 and MDM2 after p53 activation; however, there has been concordant regulation of the target gene mRNA and protein levels. For example, camptothecin induced p53-mediated elevation in p21 mRNA and protein levels and failed to increase MDM2 mRNA or protein levels (Ashcroft *et al.*, 2000). Similarly, after exposure to high dose UV, p21 mRNA and protein levels rapidly increased, while MDM2 mRNA and protein levels decreased (Wu and Levine, 1997). Thus, the protein levels of p21 and MDM2 after specific stresses is typically modulated by p53 transcriptional regulation of the respective promoters. The finding that Taxol treatment induces transcriptional upregulation of MDM2 but not an elevation in steady state MDM2 protein levels, suggests the presence of an alternative mechanism that decreases MDM2 protein levels without affecting p53 protein levels.

Several significant findings in this study resulted from experiments using the Ser-15 p53 phospho-specific antibody. For example, in contrast to the elevation in Ser-15 phosphorylated p53 observed in MTI-treated HCT116 and RKO epithelial tumor cells, the elevation in Ser-15 phosphorylated p53 in Taxol-treated NHDFs and ATM-deficient fibroblasts was only reflective of the increase in total p53 protein. These results suggest that the non-transformed fibroblasts and transformed epithelial tumor cells examined in this study induce distinct signaling pathways after microtubule disruption, resulting in differential phosphorylation of p53.

Adding a further layer of complexity to interpretation of the biological consequences of p53 phosphorylation are recent studies indicating that there is interplay between the phospho-sites, such that phosphorylation on one residue may require phosphorylation on a prior site. For example, Bulavin *et al.* (1999) observed that substitution of alanine at Ser-33 completely blocked UV-induced phosphorylation at Ser-37 but did not decrease phosphorylation at Ser-15. In contrast, the S33A/S46A double mutant abrogated UV-induced phosphorylation of Ser-37, and significantly reduced phosphorylation at Ser-15 (Bulavin *et al.*, 1999). This previous finding suggests that either the presence of Ser-33 and Ser-46 residues, or their ability to be phosphorylated, is important for amino terminal phosphorylation of p53 after cells are exposed to UV light. Similarly, Sakaguchi *et al.* (2000) and Dumaz *et al.* (1999) recently reported that IR-induced phosphorylation of p53 on Thr-18 required prior phosphorylation on Ser-15. The results from the current study are consistent with these latter studies, as the S15A/T18A double mutant was not significantly phosphorylated after microtubule disruption or genotoxic stress, in contrast to the T18A single point mutant protein that was phosphorylated after nocodazole, IR and ADR treatments.

Currently, a major challenge in the p53 field is linking biochemical modifications of the protein with biological effects. The studies cited above and the results presented in the current study illustrate the need for higher resolution techniques that will allow analysis of p53 after cellular stress. Mass spectrometric analysis of the p53 protein was recently used by Abraham *et al.* (2000) to identify p53 sites that are covalently modified

in vivo, either constitutively or in response to IR. By identifying specific post-translational modifications after cellular stress, the biochemical activity of differentially modified p53 molecules at specific promoters can begin to be evaluated. For example, co-analysis of p53 by mass spectroscopy and *in vivo* promoter-trapping (Murphy *et al.*, 1999; Szak and Pietenpol, submitted) after a specific cellular stress may allow important insight to the p53 post-translational modifications required for a biological endpoint.

Since drugs that alter microtubule dynamics such as Taxol are important anticancer agents, identifying the post-translational modifications that mediate elevation of p53 protein levels after microtubule disruption in tumor cells may have important therapeutic implications. The results from this study indicate that MTI treatment of epithelial tumor cells results in distinct patterns of phosphorylation as compared to genotoxic stress. However, we cannot rule out that microtubule disruption leads to DNA alterations in cells. Thus, the p53 phospho-forms observed may be due to DNA damage signaling pathways distinct from those induced by the genotoxic agents examined in this study. The elucidation of kinases that regulate phosphorylation of p53 after MTI treatment may ultimately identify novel targets for chemotherapeutic intervention. For example, loss of p53 function in tumor cells can enhance cellular sensitivity to MTIs (Stewart *et al.*, 1999b). Thus, the development of compounds which inhibit kinases that phosphorylate and activate p53 after microtubule disruption may enhance therapeutic efficacy when these compounds are combined with MTIs to treat tumors with intact p53 signaling pathways.

Materials and methods

Cell culture and treatment

The HCT116 and RKO human colorectal carcinoma cell lines were obtained from American Type Culture Collection (ATCC) and were maintained in monolayer culture in McCoys 5A modified medium (Gibco–BRL) supplemented with 10% fetal bovine serum (FBS; Gemini Bio-Products, Inc.), and 1% penicillin/streptomycin (Sigma). H1299 human large cell lung carcinoma cells (ATCC) have a partial homozygous deletion of the p53 gene, and p53 protein expression is not detectable (Mitsudomi *et al.*, 1992). H1299 cells were maintained in monolayer culture in F-12 medium (Sigma) supplemented with 10% FBS and 1% penicillin/streptomycin. Normal human dermal fibroblasts (NHDFs) (Vanderbilt University Research Center) were maintained in monolayer culture in Dulbecco's Modified Eagle Medium-Hi Glucose (Gibco–BRL) supplemented with 10% FBS and 1% penicillin/streptomycin. The GM02052C (AT-1) fibroblast cell line derived from AT patients (Coriell Institute for Medical Research) was maintained in monolayer culture in Minimum Essential Medium with Earle's salts (Hyclone) supplemented with 15% FBS, 1% penicillin/streptomycin, 2 mM non-essential amino acids (Hyclone), 50 μg/ml insulin-transferrin-selenium A (Boehringer Mannheim), 1 mM MEM amino acids (Gibco–BRL), and MEM vitamins (Gibco–BRL). All cells were grown at 37°C with 5% CO_2 in a humidified incubator.

When indicated, cells were treated with nocodazole (Sigma), paclitaxel (Taxol; Sigma), or Vincristine (Eli Lilly and Co.) diluted in dimethyl sulfoxide and added directly to cell media. Cells were also treated with adriamycin (ADR) (Gensia Sicor Inc.) diluted in media, ionizing radiation (IR), or ultraviolet radiation (UV) when indicated. IR was delivered at room temperature with a ^{137}Cs irradiator (JL Shepherd and Associates). UV was delivered at room temperature with a UV Stratalinker (Stratagene Cloning Systems).

Western analysis

Cell lysates were prepared and transferred onto membranes as previously described (Stewart *et al.*, 1999a). Membranes were incubated with mouse monoclonal antibodies against p53 (PAb1801), p21 (EA10) (Oncogene Research Products), and MDM2 (SMP14) (Santa Cruz Biotechnology); a rabbit polyclonal antibody against serine-15 phosphorylated p53 (New England Biolabs); and a goat polyclonal antibody against actin (I-19) (Santa Cruz Biotechnology). Primary antibodies were detected using goat α-mouse, goat α-rabbit, or rabbit α-goat horseradish peroxidase-conjugated secondary antibodies (Pierce) and enhanced chemiluminescence detection.

Northern analysis

mRNA was isolated from control and MTI-treated HCT116 cells, resolved by agarose gel electrophoresis, and transferred to nitrocellulose membrane (Gibco–BRL) as previously described (Flatt *et al.*, 2000). Human MDM2, p21, and GADPH cDNAs were labeled with α-^{32}P-dCTP using Rediprime II (Amersham). After a 2 h prehybridization incubation in Express Hyb (Clontech Laboratories, Inc.) at 50°C, membranes were incubated with 1×10^6 c.p.m. of labeled cDNA per ml in Express Hyb at 50°C overnight. Membranes were washed twice in 2 × SSC and 0.1% SDS (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 50°C, followed by two washes in 0.2 × SSC and 0.1% SDS at 50°C. The hybridized ^{32}P-labeled cDNAs were used to quantify mRNA levels on an Instant Imager (Packard Instrument) prior to autoradiography.

Two dimensional gel electrophoresis

For two dimensional (2D) gel analyses of endogenous p53 in HCT116 cells, 1 mg of protein lysate from control cells or 150 μg of protein lysate from treated cells were prepared in a final volume of 600 μl of isoelectric focusing (IEF) sample buffer (9.5 M urea (Pharmacia), 2% NP-40, 2% β-mercaptoethanol, 0.2% ampholytes pH 5-8 (Pharmacia), 0.001% bromophenol blue). For 2D analyses of p53 ectopically expressed in H1299 cells, 75 μg of protein lysate were prepared in a final volume of 600 μl of IEF sample buffer. A truncated form of recombinant human p53 (amino acids 1–353) was incubated in each sample as an internal standard to permit alignment of p53 phospho-forms; the truncated human p53 was prepared as previously described (Szak and Pietenpol, 1999). IEF was performed using the PROTEAN IEF system (Biorad) and 17 cm isoelectric strip gels pH 5-8 (Biorad). The isoelectric gels were passively rehydrated for 6 h in IEF sample buffer containing the protein lysate prior to focusing for 60 000 volt-hours. After IEF, gels were incubated for 15 min in equilibration buffer I (6M urea, 2% SDS, 0.375 M Tris (pH 8.8), 20% glycerol, 130 mM DTT) and 15 min in equilibration buffer II (6M urea, 2% SDS, 0.375 M Tris (pH 8.8), 20% glycerol, 135 mM iodoacetamide

(Aldrich Chemical Company, Inc.)) prior to separation by SDS–PAGE.

Potato acid phosphatase treatment of protein lysates

HCT116 protein lysates (150 μg) were incubated in 40 mM PIPES pH 6.0 (final volume 100 μl) for 10 min at 30°C, followed by addition of 2.0 units of potato acid phosphatases (Boehringer Mannheim). The incubation was continued for 30 min at 30°C. As a control for non-specific proteolytic degradation, protein lysates were also incubated in the absence of phosphatase under the same conditions. Phosphatase reactions were stopped by addition of Laemmli SDS–PAGE or IEF sample buffer.

Expression of p53 phosphorylation-site mutants

A panel of p53 phosphorylation-site mutants containing either alanine or aspartic acid substitutions for the indicated residues and the pCB6+ parental vector were a generous gift from Karen Vousden (National Cancer Institute, Frederick, MD, U.S.A.) (Ashcroft *et al.*, 1999). The p53 mutants include: serine-15 (S15A), threonine-18 (T18A and T18D), serine-20 (S20A), serine-37 (S37A), and a combination mutant with N-terminal and C-terminal phosphorylation sites modified to alanine (S6A, S9A, S15A, T18A, S20A, S33A, S37A, S315A, S371A, S376A, S378A, S392A). The double p53 phosphorylation-site mutant S15A/T18A in the retroviral vector pLXSN was a generous gift of Peter Herrlich (Institut für Genetik, Karlsruhe, Germany) (Blattner *et al.*, 1999). The cDNAs encoding wt p53 (*Kpn*I–*Bam*HI fragment) and the S15A/T18A mutant protein (*Eco*RI fragment) were subcloned into the pCB6+ vector. All expression vectors were verified by sequencing.

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CHAPTER 10

G2 Checkpoint and Anticancer Therapy

Zoe A. Stewart and Jennifer A. Pietenpol

Over the past two decades, the basic molecular events controlling eukaryotic G2 to M-phase cell cycle transition have been deciphered. Studies in a variety of organisms have identified an evolutionarily conserved system for controlling mitotic onset through regulation of Cdc2 kinase activity. Recently, investigations have focused on how the signaling pathways that mediate the G2 transition are regulated and modified after cellular stresses. In response to DNA damage, eukaryotic cells activate biochemical pathways, called checkpoints, to halt cell cycle progression and allow cellular damage to be repaired. Recent studies suggest that the DNA damage-induced G2 checkpoint is comprised of an early activation stage as well as a subsequent maintenance phase. In the absence of proper G2 checkpoint function, cells proceed to mitosis with damaged DNA, resulting in either apoptosis or permanent alteration of the genome that may contribute to tumorigenesis. The ability to manipulate G2 checkpoint signaling also has important clinical implications, as abrogation of the G2 checkpoint in human tumor cells can enhance cellular sensitivity to chemotherapeutic regimens that induce DNA damage. This Chapter will focus on

(i) eukaryotic DNA damage-induced G2 checkpoint signaling pathways and
(ii) how knowledge of these signaling pathways may lead to more efficient use of current anticancer therapies and the development of novel agents.

Introduction

The G2/M transition is regulated by the cyclin A/Cdc2 and cyclin B/Cdc2 kinase complexes.[1] Like other Cdk molecules, Cdc2 function is tightly regulated by a complex system involving post-translational modifications and protein-protein interactions.[2] Cdc2 activation requires association with a cyclin partner, phosphorylation on Thr-161 by CAK,[3,4] and dephosphorylation on Thr-14 and Tyr-15 by a Cdc25 phosphatase.[5,6] Several substrate proteins of Cdc2 have been identified, including the nuclear lamins A and B,[7] histone H1,[8] survivin,[9] and microtubule-associated proteins such as MAP4,[10] Eg5,[11] and stathmin.[12]

As previously noted, Cdc2 can interact with both cyclin A and cyclin B family members to regulate the G2/M transition. In mammalian cells, two cyclin A family members have been identified, cyclin A1 and cyclin A2. Mammalian cyclin A1 expression is restricted to the testis, brain, and hematopoietic cells.[13,14] Mice deficient for cyclin A1 develop normally but males are sterile due to defective spermatogenesis from impaired Cdc2 activation.[15,16] Cyclin A1 is frequently overexpressed in acute myelocytic leukemias, presumably through activation of the cyclin A1 promoter by fusion proteins created through chromosomal translocation.[13,17] Cyclin A2 is ubiquitously expressed and participates in both the G1/S and G2/M transitions by interacting with Cdk2 and Cdc2.[18,19] Homozygous deletion of cyclin A2 in mice results in embryonic lethality, demonstrating the importance of this cyclin for normal development.[20] However, further studies are necessary to define the role of cyclin A2 kinase activity in G2/M transition.

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Similar to the cyclin A family, mammalian cells contain two cyclin B family members, cyclin B1[21,22] and cyclin B2;[23] however, chickens, frogs, flies, and nematode worms possess a third, more distant relative, cyclin B3.[24,25] Both mammalian cyclin B family members are frequently co-expressed in proliferating cells, although tissue-specific temporal expression of cyclin B1 and cyclin B2 has been observed in murine germ cells.[23] Further, the two cyclin B family members have distinct subcellular localizations, with cyclin B2 strictly associated with intracellular membranes[26] while cyclin B1 is found both on intracellular membranes and in the cytoplasm.[10,27] Cyclin B2-null mice develop normally and both males and females are fertile.[28] In contrast, cyclin B1-deficient mice die in utero prior to embryonic day 10.[28] In late G2 phase of the cell cycle, cyclin B1 enters the nucleus, as nuclear localization of cyclin B1 is required during the G2/M transition.[27,29] Unlike cyclin B1, cyclin B2 does not relocate to the nucleus during the G2/M transition.[26] The nuclear localization of *Xenopus* cyclin B1 is mediated by phosphorylation of residues in the cytoplasmic retention signal sequence of the protein.[29] Several studies indicate that cyclin B1 is continuously exported from the nucleus by interaction with the nuclear exporter CRM1 during interphase, as the cytoplasmic retention signal of cyclin B1 contains a functional nuclear export signal.[30-32] Since the role of cyclin B1 in the G2/M transition has been the most extensively studied and the best defined of any eukaryotic Cdc2-associated cyclin, the subsequent discussion of Cdc2 activity will focus on regulation of the cyclin B1/Cdc2 complex.

During the G2-phase of the cell cycle, inactive cyclin B1/Cdc2 complexes accumulate in mammalian cells due to inhibitory phosphorylation of Cdc2 on Thr-14 and Tyr-15 by the Wee1 and Myt1 kinases (Fig. 10.1).[33,34] Wee1 is a dual-specificity protein kinase that was initially identified as a dose-dependent inhibitor of mitosis in *S. pombe*.[35] When Cdc2 is complexed with cyclin B1, recombinant Wee1 can phosphorylate Cdc2 on Tyr-15 and inhibit Cdc2 kinase activity.[33] Wee1 is hyperphosphorylated and degraded during mitosis, suggesting that negative regulation of Wee1 is part of the mechanism that mediates Cdc2 activation during the G2/M transition.[36-38] In support of this, studies in fission yeast have shown that Nim1 negatively regulates the S. *pombe* Wee1 by phosphorylation of its C-terminal catalytic region.[39-41] Further, *Xenopus* Wee1 is inhibited by phosphorylation in its N-terminal noncatalytic region by an unidentified protein kinase.[42] However, the kinases responsible for the phosphorylation and negative regulation of Wee1 in mammalian cells have not been determined.

The Wee1-related protein kinase, Myt1, is a cytoplasmic, membrane-bound kinase found in *Xenopus* and mammalian cells that can phosphorylate Cdc2 on both Thr-14 and Tyr-15, but has a strong preference for Thr-14.[34,43,44] Human Myt1 localizes to the endoplasmic reticulum and Golgi complex [45] and specifically phosphorylates and inactivates Cdc2 complexes to inhibit G2/M progression.[46] Myt1 interacts with Cdc2 complexes through its carboxy terminus and overexpression of human Myt1 prevents entry into mitosis by sequestering cyclin B1/cdc2 complexes in the cytoplasm.[47,48] Thus, Myt1 inhibits the G2/M transition by disrupting the nuclear localization of cyclin B1/Cdc2 complexes, as well as by phosphorylating Cdc2 on Thr-14 and Tyr-15. Another Wee1-related kinase, Mik1, has been identified in fission yeast. Mik1 acts redundantly with Wee1 in *S. pombe* to negatively regulate Cdc2 through phosphorylation of Tyr-15.[49] While a null allele of Mik1 has no discernible phenotype in fission yeast, a Mik1/Wee1 double mutant undergoes mitotic lethality that is correlated with loss of tyrosine phosphorylation on Cdc2.[49]

In eukaryotic cells, the Cdc25 family of dual-specificity phosphatases dephosphorylates Cdks to mediate Cdk activation and cell cycle progression. Mammalian cells contain three isoforms of Cdc25 (A, B, and C) that share 40-50% amino acid identity and regulate distinct cyclin/Cdk complexes throughout the cell cycle.[50,51] Cdc25A dephosphorylates cyclin E/Cdk2 and cyclin A/Cdk2 complexes to regulate the G1 and S-phase progression.[52] Cdc25B activity peaks during the G2-phase and ablation of Cdc25B activity prevents mitotic entry, suggesting Cdc25B is a positive mitotic regulator.[53-55] Further, Cdc25B dephosphorylates Cdc2 on Thr-14 and Tyr-15 *in vitro*[6] and overexpression of Cdc25B causes cells to prematurely enter mitosis.[56]

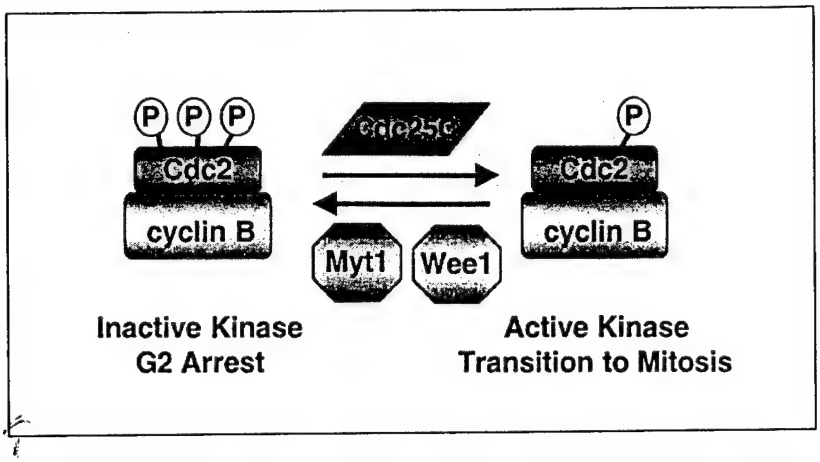


Fig. 10.1. G2/M Transition. The activity of Cdc2 is tightly regulated by phosphorylation and protein interactions. Cdc2 activation requires association with cyclin partners and phosphorylation by CAK. During S-and G2-phases, cells accumulate cyclin B1/Cdc2 in an inactive form due to inhibitory phosphorylations by Wee1 and Myt1 kinases. The conversion of Cdc2 from an inactive to active form is mediated by the Cdc25C phosphatase. Mitotic events are stimulated by active cyclin B/Cdc2 complexes.

Several studies implicate Cdc25A and Cdc25B as human oncogenes, as overexpression of these proteins is observed in primary breast tumors as well as head and neck cancers.[57,58]

Cdc25C is required for entry into mitosis and is believed to be the major phosphatase that dephosphorylates Thr-14 and Tyr-15 of Cdc2 *in vivo*.[5] In cycling *Xenopus* egg extracts, the activity of Cdc25 (biochemically equivalent to human Cdc25C) is low during S-phase and increases in mitosis,[51] and recombinant human Cdc25C dephosphorylates Cdc2 in vitro.[50,59] *Xenopus* Cdc25 is extensively phosphorylated in its N-terminal noncatalytic region during mitosis and active cyclin B/Cdc2 activates Cdc25 by phosphorylating the N-terminal region of Cdc25 in an autocatalytic loop.[60,61] Thus only a small amount of active cyclin B/Cdc2 is required for rapid activation of all the cyclin B/Cdc2 in the cell. In addition to cyclin B/Cdc2, other protein kinases are likely to be important for Cdc25 activation; these kinases may mediate the initial activation of cyclin B/Cdc2 during G2/M.[62] For example, the polo-like kinase, Plx1, is associated with Cdc25 in *Xenopus* egg extracts and phosphorylates Cdc25 on its amino terminus to stimulate its activity.[63] The mammalian Plx1 homolog, Plk1, also phosphorylates Cdc25C, and Plk1 activity is necessary for the functional maturation of centrosomes in late G2/early prophase and for the establishment of a bipolar spindle.[64,65] Plk1 may be a useful prognostic marker for head and neck,[66] esophageal,[67] and non-small cell lung[68] carcinomas, as overexpression in these tumors is correlated with poorer prognosis.

G2 Checkpoint Activation

At key transitions during eukaryotic cell cycle progression, signaling pathways monitor the successful completion of upstream events prior to proceeding to the next phase. These regulatory pathways are commonly referred to as cell cycle checkpoints.[69] Cells can arrest at cell cycle checkpoints temporarily to allow for

(1) the repair of cellular damage,
(2) the dissipation of exogenous cellular stressors, or
(3) the accumulation of essential nutrients and growth factors. Checkpoint signaling may also activate pathways leading to programmed cell death if cellular damage cannot be properly re-

paired. Defects in cell cycle checkpoints can result in gene mutations, chromosome damage, and aneuploidy, all of which result in permanent alteration of the genome.[70] The checkpoint mechanisms that normally regulate the fidelity of cell cycle progression are frequently disrupted in tumor cells, verifying the importance of intact checkpoint signaling pathways for maintenance of the genome.[71]

In addition to controlling normal mitotic entry, regulation of Cdc2 phosphorylation plays a critical role in activating the G2 checkpoint after DNA damage. Genotoxic stress activates cell cycle checkpoint pathways that initiate DNA repair and arrest at the G2/M transition to prevent the propagation of DNA lesions during mitosis.[72] Initial experiments in the fission yeast *S. pombe* demonstrated that phosphorylation of Cdc2 on Tyr-15 is required for G2 arrest after ionizing radiation treatment.[73] Expression of a dominant Cdc2 mutant (Tyr-15 changed to phenylalanine) completely abolishes the mitotic delay observed after ionizing radiation exposure.[73] The mechanism by which Tyr-15 phosphorylation is upregulated in fission yeast after DNA damage involves both increased Wee1 protein levels and kinase activity, as well as decreased Cdc25 (biochemical equivalent of human Cdc25C) levels and activity.[73-75] The importance of this two-step mechanism to modulate Tyr-15 phosphorylation of Cdc2 is evidenced by the finding that while Wee1 inactivation in *S. pombe* increases sensitivity to both ionizing[76] and ultraviolet[77] radiation, Wee1-deficient yeast maintain G2 checkpoint control.[78] In contrast, inactivation of both Wee1- and Cdc25-dependent function completely abolishes checkpoint control in addition to sensitizing cells to ionizing and ultraviolet (UV) radiation.[75] Thus, upregulation of Cdc2 Tyr-15 phosphorylation after DNA damage requires both increased phosphorylation of Tyr-15 and a reduced rate of Tyr-15 dephosphorylation.[75] Recent studies indicate that Mik1 may also function during the G2 DNA damage checkpoint in fission yeast, as Mik1 protein levels are induced in response to ionizing radiation.[79,80]

Similar mechanisms appear to govern the DNA damage-induced G2 checkpoint arrest in mammalian cells, as phosphorylation of Cdc2 on Thr-14 and Tyr-15 plays a critical role in the DNA damage-induced G2 arrest (Fig. 10.2).[81] Treatment of Chinese hamster ovary cells with either etoposide or ionizing radiation results in rapid Cdc2 Tyr-15 phosphorylation and inhibition of cyclin B/Cdc2 kinase activity.[82,83] Increased Tyr-15 phosphorylation of Cdc2 is observed in HL-60 human myeloid leukemia cells after exposure to ionizing radiation.[84] Further, the overexpression of a non-phosphorylatable Cdc2 mutant (Thr-14 and Tyr-15 mutated to alanine and phenylalanine, respectively), significantly reduces the G2 delay observed in HeLa cells exposed to ionizing radiation.[85] Numerous studies have also shown that after genotoxic stress, the G2 checkpoint can be the predominant phase of cell cycle arrest in primary epithelial cells, including keratinocytes[86] as well as prostate,[87] and bronchial epithelial cells.[88]

While the biochemical pathways involved in the DNA damage-induced G2 arrest in mammalian cells entail signaling cascades that converge to inhibit Cdc2 activation[72], specific pathways that mediate G2 arrest after DNA damage in higher eukaryotes have only recently been elucidated. In human hematopoietic cells, ionizing radiation activates the Lyn kinase, a member of the Src family of protein tyrosine kinases.[89] Lyn localizes to the nucleus and directly binds to Cdc2 in hematopoietic cells exposed to ionizing radiation.[90] Further, recombinant Lyn phosphorylates Cdc2 on Tyr-15 in vitro, suggesting this kinase may be important in the G2 checkpoint response in hematopoietic cells.[90,91] In addition to upregulation of Cdc Tyr-15 phosphorylation, decreased dephosphorylation of Cdc2 may also be important in G2 arrest in mammalian cells, as impaired Cdc25C activation is observed in human lymphoma cells treated with nitrogen mustard,[92] as well as in HeLa cells treated with either ionizing[93] or UV [94] radiation.

Recent studies have identified critical evolutionarily conserved G2 checkpoint pathways mediated by members of the phosphoinositide-3 kinase (PI-3K) family in response to genotoxic stress. After DNA damage, the PI-3K family members ATM (Tel1 in fission and budding yeast) and ATR (Rad3 and Mec1 in fission and budding yeast, respectively), become activated and initiate specific signal transduction pathways that regulate DNA repair and cell cycle arrest in yeast, *Xenopus*, and mammalian cells (Fig. 10.2). ATM phosphorylates and activates the

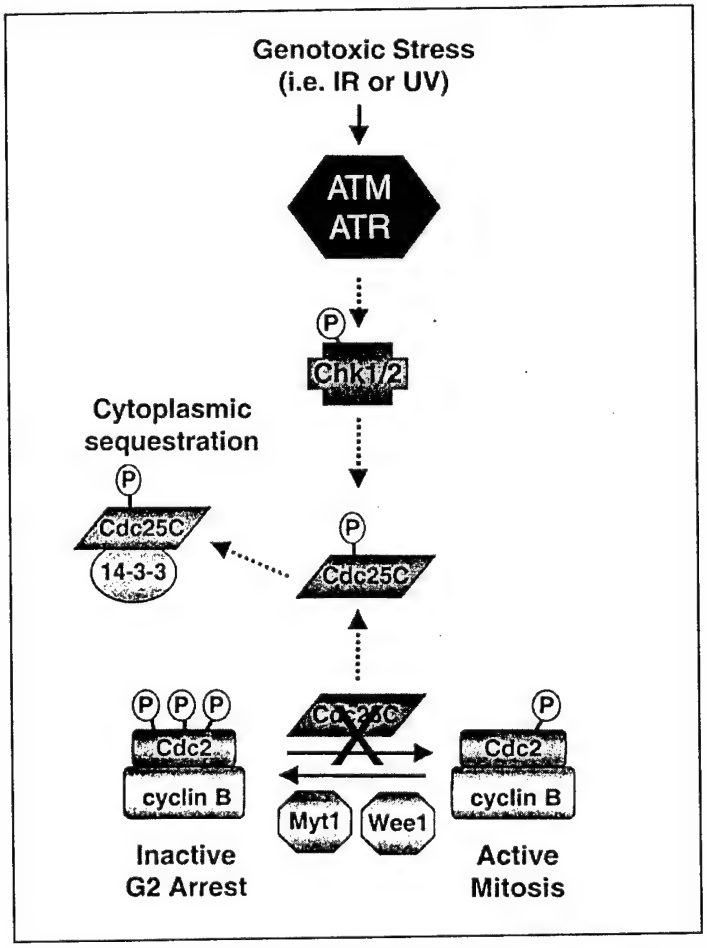
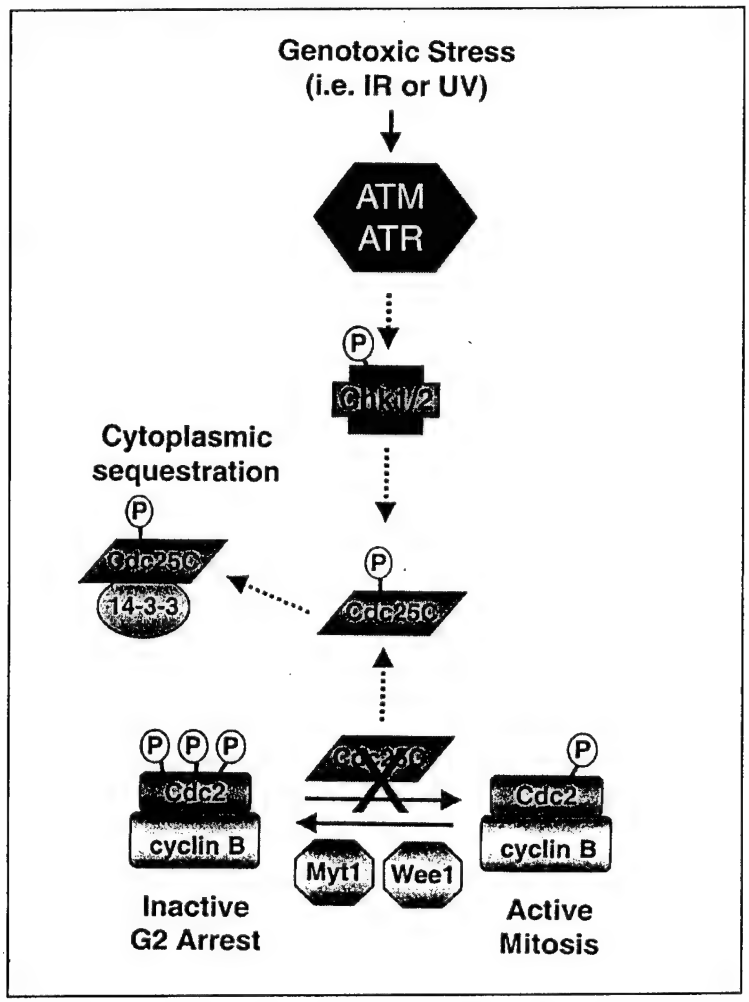


Fig. 10.2. G2 Checkpoint Activation After Genotoxic Stress. In response to genotoxic stress, the ATM/ATR signaling pathway is activated leading to phosphorylation and activation of Chk1 and Chk2 kinases and subsequent phosphorylation of Cdc25C. This latter phosphorylation promotes the interaction of Cdc25C with 14-3-3 adaptor proteins and inhibits the ability of Cdc25C to activate cyclin B1/Cdc2, resulting in G2 cell cycle arrest.

Chk2 kinase (Cds1 and Rad53 in fission and budding yeast, respectively) in cells exposed to ionizing radiation.[95-98] Similarly, ATR-dependent signaling mediates activation of the Chk1 kinase (Chk1 in fission and budding yeast) in cells treated with UV radiation.[99,100] Activated Chk1 and Chk2 can phosphorylate Cdc25C on Ser-216, generating a consensus binding site for 14-3-3 proteins.[95,101-104] The binding of 14-3-3 proteins to Cdc25C results in the nuclear export of Cdc25C, cytoplasmic sequestration of the phosphatase, and G2 arrest due to inhibition of Cdc2 activity.[105]

In addition to phosphorylating Cdc25 on Ser-216, activated Chk1 also phosphorylates Wee1 in fission yeast, resulting in stabilization of the protein and thus prolonged Wee1 kinase



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In addition to phosphorylating Cdc25 on Ser-216, activated Chk1 also phosphorylates Wee1 in fission yeast, resulting in stabilization of the protein and thus prolonged Wee1 kinase

activity during G2 checkpoint responses.[74] Further, Chk1 is essential for the DNA damage-induced G2 checkpoint in *S. pombe* as Chk1-deficient cells have increased sensitivity to ionizing radiation due to defective G2 arrest.[106] Thus Chk1 integrates Rad3-dependent sigaling in fission yeast to mediate the two-step mechanism to modulate Tyr-15 phosphorylation of Cdc2 through activation of the Wee1 kinase and inactivation the Cdc25 phosphatase.[75]

The importance of the PI-3K G2 checkpoint pathways is exemplified by the fact that yeast and mammalian cells deficient in function of these family members have defective G2 checkpoint responses after DNA damage resulting in enhanced cellular sensitivity to genotoxic stressors. Inactivation of Rad3[107,108] and Mec1[109] in yeast cells ablates G2 arrest after ionizing radiation, while the expression of a kinase-defective ATR in human fibroblasts abrogates the G2 checkpoint and sensitizes the cells to both ionizing and UV radiation.[110] Further, homozygous deletion of ATR in mice results in embryonic lethality, as cells die by chromosome fragmentation and apoptosis.[111,112]

There are overlapping functions between the ATR and ATM classes of PI-3K kinases in yeast. Deletion of the ATM homolog, Tel1, in fission[113] or budding[114] yeast has minimal effect on DNA damage-induced checkpoint responses, as Tel1 functions to maintain telomere integrity in yeast.[115] In contrast, loss of ATM in mammalian cells eliminates G2 checkpoint function and sensitizes cells to ionizing radiation,[116,117] but does not alter checkpoint maintenance or cellular sensitivity to UV radiation. ATM-null mice exhibit growth retardation, neurologic dysfunction, infertility, defective T lymphocyte maturation, and sensitivity to ionizing radiation.[117,118] The majority of ATM-deficient animals develop malignant lymphomas by 4 months of age, while ATM-/- fibroblasts have abnormal radiation checkpoint function after exposure to ionizing radiation.[117,118] ATM function is defective in patients with ataxia telangiectasia, a disorder in which patients have increased sensitivity to radiation[119] and are highly prone to the development of leukemias and lymphomas in childhood.[120] Further, individuals harboring a heterozygous germ line mutation of ATM have an elevated incidence of breast cancer.[120,121]

Chk1 and Chk2 proteins are also essential for maintenance of the DNA damage-induced G2 checkpoint in yeast and mammalian cells. Loss of Chk1 in fission yeast,[106] budding yeast,[122] and mammalian cells.[99,123] results in defective G2 checkpoint function after exposure to ionizing radiation. Abrogation of Chk1 in mice results in early embryonic lethality.[99,123] Similarly, Cds1[124] and Rad53[125,126] are essential for G2 checkpoint integrity after DNA damage in fission and budding yeast, respectively. Mammalian Chk2-/- embryonic stem cells also fail to undergo G2 arrest after ionizing radiation treatment, while Chk2-/- thymocytes are resistant to DNA damage-induced apoptosis.[127] Of note, heterozygous germline mutations of Chk2 occur in a subset of individuals with Li-Fraumeni syndrome, a highly penetrant familial cancer syndrome associated with significantly increased rates of brain tumors, breast cancers, and sarcomas that is typically associated with germline mutations in the tumor suppressor p53.[128]

While regulation of Cdc25C by PI-3K family members plays a critical role during G2 checkpoint responses, other kinases are capable of phosphorylating Cdc25C on Ser-216 in response to DNA damage in mammalian cells. For example, C-TAK1 is a kinase that is ubiquitously expressed in human cells and phosphorylates Cdc25C on Ser-216 to promote 14-3-3 protein binding.[129] Similarly, Prk is a kinase expressed in human ovary, placenta, and lung that also phosphorylates Cdc25C on Ser-216 in vitro.[130-132] Prk mRNA expression is downregulated in human lung tumors, suggesting that disruption of specific G2 checkpoint pathways may contribute to tumorigenesis.[130] However, a role for C-TAK1 and Prk-dependent phosphorylation of Cdc25C after DNA damage has not been established.

Recently, the Plk1 kinase (Cdc5 in fission and budding yeast) was linked to G2 DNA damage checkpoint signaling. Smits et al[65] reported that Plk1 activity is inhibited in the G2 phase of the cell cycle in human tumor cells exposed to ionizing radiation, camptothecin, and doxorubicin. The fission[133,134] and budding[135] yeast homologs of mammalian Plk, Cdc5, also participate in G2 checkpoint signaling by preventing anaphase entry and mitotic exit after DNA damage. Expression of a mutant Plk1 in which residues necessary for Plk1 activation are

altered, prevents Plk1 inactivation and leads to G2 override in cells treated with doxorubicin.[65] Similarly, Plk1 activity is persistent during abrogation of the G2 checkpoint in tumor cells by caffeine treatment.[65] Several independent studies demonstrate that normal epithelial cells[136] and fibroblasts[64] undergo a G2 arrest in response to Plk inactivation in the absence of DNA damage. In contrast, inhibition of Plk in human tumor cells under the same circumstances results in mitotic catastrophe, although this latter event is independent of Plk1-mediated phosphorylation and inactivation of Cdc25.[64,136]

In budding yeast, *Xenopus*, and mammalian cells, the G2 checkpoint response may also be regulated by the Pin1 protein. Pin1 is an essential peptidyl-prolyl isomerase that inhibits entry into mitosis and is also necessary for mitotic progression.[137] Depletion of Pin1 from yeast or mammalian cells induces mitotic arrest, while Pin1 overexpression in these cells results in a G2 arrest.[137] In both *Xenopus* and human cells, Pin1 directly interacts with Wee1, Myt1, Cdc25C, and Plk1 in a phosphorylation-dependent manner.[138,139] The binding of Pin1 to Cdc25C inhibits the phosphatase activity of the latter, thus accounting for the ability of Pin1 to block mitotic entry.[138] Further, Winkler et al[140] determined that Pin1 is essential for DNA replication checkpoint responses, as depletion of Pin1 from *Xenopus* extracts results in inappropriate G2 progression and mitotic entry in the presence of aphidocolin. Depletion of Pin1 activity from human tumor cells by multiple mechanisms, including overexpression of Pin1 antisense mRNA, overexpression of a dominant-negative Pin1, and treatment of cells with a chemical inhibitor of Pin1, juglone, confirms that Pin1 catalytic activity is essential for both tumor cell survival and mitotic entry.[141] However, further studies are necessary to determine if Pin1 function is required for the DNA damage-induced G2 checkpoint.

Proteins that mediate direct repair or detection of DNA damage may also be essential for proper G2 checkpoint activation. MLH1 is a necessary component of the DNA mismatch repair machinery and is thought to participate in the G2 checkpoint in human tumor cells. Ovarian tumor cells lacking MLH1 expression have defective G2 cell cycle arrest after cisplatin and 6-thioguanine treatment.[142] Further, MLH1-deficient human colon carcinoma cells also have decreased survival and concomitant G2 checkpoint deficiency after ionizing radiation or 6-thioguanine exposure as compared to genetically matched cells in which mismatch repair function has been restored.[143] Similarly, MLH1-/- mouse embryo fibroblasts (MEFs) are sensitized to ionizing radiation and 6-thioguanine and have impaired G2 arrest after exposure to these agents as compared to wild-type (wt) MEFs.[143] Interestingly, Brown et al[142] observed loss of MLH1 expression in 9 of 10 human ovarian cell lines after cisplatin treatment in vitro, as well as the loss of MLH1 expression in 4 of 11 tumors biopsied during second look laparotomy after chemotherapy. Since, MLH1-deficiency in human ovarian cells renders them resistant to numerous chemotherapeutic agents, including cisplatin, doxorubicin, 6-thioguanine, and N-methyl-N-nitrosourea, these latter findings have important therapeutic implications.[142] Taken together, these studies suggest that MLH1-mediated regulation of the G2 checkpoint is indispensable for proper DNA damage detection and repair, although the mechanism by which MLH1 enforces G2 checkpoint integrity has not been elucidated.

Members of the homeobox family of proteins have also been implicated in regulation of the G2 checkpoint. HSIX1 is a homeobox protein expressed during the S and G2 phases of the cell cycle.[144] Overexpression of HSIX1 in human breast cancer cells abrogates the G2 cell cycle checkpoint response after ionizing radiation.[144] Further, HSIX1 expression is absent or very low in normal mammary tissue but is elevated in nearly half of primary breast cancers and 90% of metastatic lesions.[144] HSIX1 expression is also elevated in a variety of human cancer cell lines, suggesting an important function for the protein in multiple tumor types.[144] Further studies are necessary to determine if HSIX1 function is mediated through regulation of the G2 checkpoint.

Finally, several members of the MAPK family are activated in response to ionizing radiation; however, the biological relevance of these kinases to the G2 checkpoint is uncertain. Recently, the p38γ kinase was shown to have an essential function in the G2 checkpoint in human tumor

cells exposed to ionizing radiation.[145] Activation of p38γ occurs in tumor cells treated with cisplatin, etoposide, or ionizing radiation.[145,146] Further, p38γ-dependent signaling is required for DNA damage-induced G2 arrest, as disruption of p38γ-mediated signaling abrogates the G2 arrest and enhances the cytotoxicity observed in human tumor cells and human fibroblasts treated with ionizing radiation.[145] The DNA damage-mediated activation of p38γ is ATM-dependent. ATM-deficient cells fail to induce p38γ activity after ionizing radiation treatment; however, the downstream targets of p38γ activity during G2 checkpoint signaling have not been elucidated.[145]

G2 Checkpoint Maintenance

Numerous studies indicate that the G2 arrest response is comprised of an early or activation stage as well as a subsequent maintenance phase, with p53 signaling implicated to play a role in the latter.[147-150] While the importance of p53-mediated signaling in G1/S checkpoint function is well documented, the role of p53 signaling at the G2 checkpoint has only recently been well defined. Early studies showing p53-deficient cells maintain the DNA damage-induced G2 arrest suggested that p53 does not function to regulate the G2 checkpoint.[151,152] However, expression of p53, in the absence of cellular stress, induces cell cycle arrest at both the G1 and G2 checkpoints, suggesting p53 signaling modulates the G2 checkpoint response.[153-155] Subsequent studies showed that p53 and $p21^{Waf1/Cip1}$ (p21) are necessary to maintain a G2 arrest following DNA damage, since tumor cells lacking these proteins enter into mitosis with accelerated kinetics.[150,156]

p53 utilizes multiple signaling pathways to modulate the G2 checkpoint (Fig. 10.3). One of the initial components of p53-dependent G2 checkpoint maintenance is the transcriptional upregulation of 14-3-3σ. 14-3-3σ is induced in a p53-dependent manner by exposure to ionizing radiation and doxorubicin in colorectal carcinoma cells.[157] Further, overexpression of 14-3-3σ in proliferating cells induces a G2 arrest.[157] Deletion of both alleles of 14-3-3σ in colorectal carcinoma cells results in abrogation of G2 arrest and premature mitotic entry after exposure to ionizing radiation and doxorubicin.[158] The p53-dependent increase in 14-3-3σ is thought to modulate cyclin B1/Cdc2 signaling, as the binding of 14-3-3σ to Cdc2 results in cytoplasmic sequestration of the kinase.[158]

In addition to transcriptional upregulation of 14-3-3σ, the mechanism of p53-dependent G2 arrest involves inhibition of cyclin B1/Cdc2 activity by p21 and a subsequent reduction of cyclin B1 and Cdc2 protein levels.[156,159-161] Similar to its regulation of the cyclin E/Cdk2 complex at the G1/S checkpoint, p21 binds to and inhibits the cyclin B1/Cdc2 complex in vitro by blocking the activating phosphorylation of Cdc2 on Thr-161,[162] although p21 has a significantly lower affinity for the cyclin B1/Cdc2 complex as compared to the G1 phase kinase complexes.[163] Thus, DNA damage-induced G2 delay is regulated by modulation of both the activating and inhibitory phosphorylations of Cdc2. The reduced expression of cyclin B1/Cdc2 is mediated in part by p53-dependent transcriptional repression of the cyclin B1 and Cdc2 promoters, although this transrepression is not due to direct interaction of p53 with these promoters.[156,164] The CCAAT-biding factor NF-Y was recently shown to mediate transcriptional inhibition of cyclin B1 and Cdc2 during p53-dependent G2 arrest.[165] Cdc2 transrepression may also result from the interaction of p130 and E2F4 with the Cdc2 promoter.[166] The importance of p53-dependent regulation of Cdc2 activity is exemplified by the finding that constitutive activation of cyclin B1/Cdc2 overrides p53-mediated G2 arrest.[167]

The p53-mediated decrease in cyclin B1 and Cdc2 transcription also requires the retinoblastoma protein (pRB). Abrogation of pRB function in cells containing wt p53 blocks the down regulation of cyclin B1 and Cdc2 expression and leads to an accelerated exit from G2 after genotoxic stress.[156] Thus, similar to what occurs in cells that are p21 and p53 deficient, pRB loss can uncouple S phase and mitosis after genotoxic stress in tumor cells. pRB is a transcription repressor that, in its hypophosphorylated state, binds to the E2F-family (E2F) of transcription factors and blocks E2F-dependent transcription of genes whose products are nec-

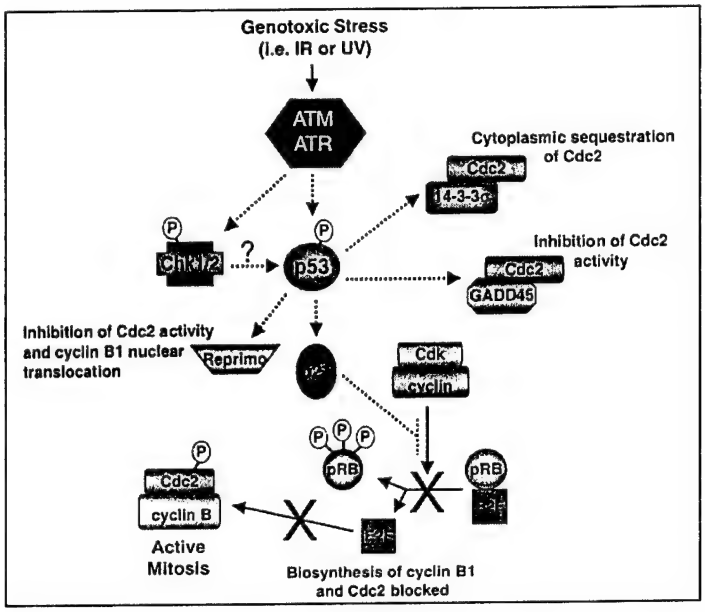

Figure 3. G2 Checkpoint Maintenance After Genotoxic Stress. After DNA damage, activated ATM/ATR as well as Chk1 and Chk2 can phosphorylate p53, resulting in stabilization and activation of the tumor suppressor. p53-dependent signaling contributes to maintenance of the G2 cell cycle arrest by upregulating the 14-3-3σ protein that binds to Cdc2 and sequesters the kinase in the cytoplasm. p53-dependent transcription also elevates the Cdk inhibitor p21, that binds to cyclin/Cdk complexes to reduce phosphorylation of pRB. Hypophosphorylated pRB remains bound to E2F, preventing E2F from mediating the biosynthesis of cyclin B1 and Cdc2. p53 may also play a role in G2 checkpoint maintenance through transcriptional upregulation of GADD45 and Reprimo. GADD45 can directly impede cyclin B1/Cdc2 activity after UV radiation by binding to Cdc2. Reprimo can inhibit Cdc2 activity and inhibit nuclear translocation of cyclin B1.

essary for S-phase entry, G2 progression, and M phase transition.[168] Activated G1-phase cyclin/Cdk complexes phosphorylate pRB, resulting in the dissociation of E2F and pRB and cell cycle progression after E2F-mediated transcription.

In addition to modulation of cyclin B1/Cdc2 activity by indirect transcriptional mechanisms, p53 may also exert G2 checkpoint responses through transcriptional upregulation of the downstream target genes, GADD45, and Reprimo. Increased expression of GADD45 in primary fibroblasts results in a G2 arrest that can be abrogated by the overexpression of cyclin B1 or Cdc25C.[169] This GADD45-induced G2 arrest is p53-dependent since overexpression of GADD45 in p53-deficient fibroblasts fails to mediate a G2 arrest.[169] Also, GADD45 has been shown to directly inhibit the cyclin B1/Cdc2 complex after UV radiation by binding to Cdc2.[170] Of note, the GADD45-dependent G2 arrest is induced only after specific types of DNA damage. Lymphocytes from GADD45 knockout mice failed to arrest after exposure to UV radiation, but retained the G2 checkpoint initiated after exposure to ionizing radiation.[169] Reprimo was recently identified as a novel p53 downstream target that is induced in MEFs exposed to ionizing radiation.[171] Reprimo is a highly glycosylated protein that induces G2 arrest when overexpressed in human tumor cells regardless of p53 status.[171] Cdc2 and cyclin B1 protein

levels are unaffected by Reprimo overexpression, although Cdc2 activity and cyclin B1 nuclear translocation are inhibited.[171]

p53-mediated transrepression of target genes may also contribute to its role in G2 arrest. One critical cell cycle target of p53 transrepression is stathmin/oncoprotein 18.[172,173] Stathmin is frequently overexpressed in breast[174,175] and ovarian[176] cancers, as well as hematologic malignancies,[177,178] suggesting a critical function in cell cycle control. Stathmin is a microtubule-associated phosphoprotein that functions in the regulation of microtubule dynamics during mitosis.[179] Inhibition of stathmin by mutation of sites required activating phosphorylations by Cdc2 and MAPK prevents mitotic spindle formation.[12,180-182] Overexpression of stathmin can override p53-dependent G2 arrest in human tumor cells exposed to ionizing radiation, indicating that regulation of stathmin expression is critical to p53-regulated G2 checkpoint responses.[173]

In addition to their previously described role in G2 checkpoint function, the ATM, ATR, Chk1, and Chk2 kinases also directly phosphorylate the amino terminus of p53 after DNA damage.[183-186] ATM-and ATR-induced phosphorylation of human p53 on Ser-15 may be important for p53 activation after genotoxic stress.[183-187] Similarly, Chk1- and Chk2-mediated phosphorylation of human p53 on Ser-20 may contribute to p53 activation after DNA damage,[127,188,189] since substitution of Ser-20 with alanine abrogates p53 stabilization after exposure to either ionizing or UV radiation.[190] Further, as previously described, cells deficient in either ATM, ATR, Chk1, or Chk2 have defective G2 arrest after ionizing radiation, a phenotype similar to that of p53-/- cells.[150] However, p53 appears to function through the G1/S checkpoint after activation by these kinases in response to DNA damage;[189] although, a role for these kinases in p53-dependent G2 checkpoint responses cannot be excluded.

The p53-mediated G2 checkpoint also modulates genomic integrity after MYC oncogene overexpression. Felsher et al[191] recently reported that overexpression of MYC protein in human fibroblasts triggers aneuploidy through a mechanism involving p53-dependent G2 arrest. Loss of the p53-mediated G2 checkpoint decreases the number of aneuploid cells after MYC overexpression, as p53 inactivation reduces the population of G2-arrested cells with the potential to become aneuploid.[191] Thus, cells in the G2 or M phases of the cell cycle become aneuploid regardless of p53 status in the presence of activated MYC; however, the loss of p53-dependent cell cycle checkpoints and p53-mediated apoptosis enhances the ability of MYC-overexpressing cells to progress through the cell cycle and become aneuploid.

Experiments analyzing viral proteins that inactivate p53 or pRB demonstrate the importance of the p53-mediated, pRB-dependent G2 checkpoint after DNA damage. p53 function is disrupted in cells by the ectopic expression of the human papillomavirus E6 protein. E6 binds p53 and targets it for ubiquitin-mediated degradation, thus abrogating p53-dependent signaling.[192] Human fibroblasts and tumor cells expressing E6 have attenuated G2 checkpoint function after ionizing radiation exposure[148,156,193,194] or adriamycin,[156,195] as a significantly greater proportion of E6-containing cells enter mitosis as compared to control cells. Similarly, loss of the G2 checkpoint is observed after exposure to ionizing radiation in fibroblasts[196] and tumor cells[156] expressing the human papillomavirus E7 protein, as the E7 protein binds to and inactivates pRB. The adenovirus E1A protein is also capable of disrupting the DNA damage-induced G2 checkpoint.[197,198] Mouse keratinocytes and human tumor cells expressing the E1A protein are sensitized to treatment with cisplatin, adriamycin, and ionizing radiation due to defective G2 arrest.[199,200] However, it remains unclear if E1A-mediated disruption of the G2 checkpoint is p53-dependent. Sanchez-Prieto et al[199] failed to find a correlation between the presence or absence of p53 in keratinocytes and the ability of E1A to impair G2 checkpoint activation. In contrast, Bulavin et al[197] found that in E1A-expressing rat fibroblasts exposed to ionizing radiation, deregulation of p53-dependent signaling pathways contributed to defective G2 delay. Regardless of its ability to modulate p53-dependent G2 checkpoint signaling, E1A directly mediates transactivation of the Cdc2 promoter to facilitate G2 progression, although

the contribution of E1A-mediated events to abrogation of the G2 checkpoint has not been elucidated.[201]

In some hematopoietic cell lines, p53 may accelerate the exit from G2 after DNA damage and this accelerated mitotic entry contributes to p53-mediated apoptosis. In murine myeloid leukemia cells bearing a temperature-sensitive p53 mutant, wt p53 positively modulates G2/M progression after etoposide or ionizing radiation exposure.[202,203] In these studies, the accelerated G2/M progression induced by p53 was associated with enhanced cytotoxicity and apoptosis. Similarly, myeloblast-enriched bone-marrow cells from p53+/+ mice have accelerated mitotic entry after ionizing radiation as compared to cells from p53-/- mice.[203] Thus, while p53 appears to be required for DNA damage-induced G2 arrest in epithelial cells, the G2 checkpoint in hematopoietic cells may be p53-independent.

Similar to hematopoietic cells, downregulation of Wee1 mRNA and protein expression and loss of Cdc2 Tyr-15 phosphorylation is observed after activation of p53 in rat embryo fibroblasts expressing the temperature-sensitive p53val135 mutant.[204] Downregulation of Wee1 also occurs in thymus of p53+/+ but not p53-/- mice after exposure of the animals to ionizing radiation.[204] The p53-mediated reduction in Wee1 is likely due to a transrepression mechanism similar to that discussed previously for cyclin B1 and Cdc2. Based upon the important roles of Wee1 and Cdc2 in regulating the G2/M transition, this mechanism may represent one biochemical pathway by which p53 modulates G2/M progression after DNA damage.

Modulation of the G2 Checkpoint—Therapeutic Implications

Since preclinical studies have shown that cells with defective checkpoint function are more vulnerable to anticancer agents, it is hypothesized that the same will hold true in the clinical setting. Indeed, numerous laboratories are now searching for compounds that interfere with and/or override cell cycle checkpoints, in hope that such agents may be more effective in anticancer therapy. A majority of tumor cells have defective G1 checkpoint function, making the G2 checkpoint their "last line of defense" after exposure to DNA damaging agents. Thus, the G2 checkpoint is a particularly attractive target for chemotherapeutic manipulation, since ablation of G2 checkpoint function in tumor cells may result in enhanced susceptibility to genotoxic anticancer drugs.

Chemical Approaches

There is strong evidence that abrogation of DNA damage-induced G2 arrest in human cancer cell lines results in higher rates of apoptosis. Exposure of cells to ionizing radiation in combination with caffeine or pentoxifylline, compounds which activate Cdc2 by activation of Cdc25C phosphatase, results in G2 checkpoint override and increased rates of apoptosis.[205-207] Caffeine disrupts the G2 checkpoint in p53-defective cells and results in radiosensitization of tumor cells;[205-207] however, the concentrations of caffeine required for abrogation of the G2 checkpoint in vitro are too cytotoxic for in vivo use. Nonetheless, elucidation of how caffeine overrides the G2 checkpoint has provided important mechanistic insight to how the G2 checkpoint can be modulated to enhance therapeutic efficacy. Caffeine inhibits the catalytic activity of both ATM and ATR at drug concentrations similar to those that induce radiosensitization.[184,208] Treatment of tumor cells with caffeine blocks ATM-mediated phosphorylation of Chk2 on Thr-68 after ionizing radiation.[209] ATR phosphorylates Chk1 on Ser-345 in human cells after ionizing and UV radiation exposure.[99] In *Xenopus*, DNA damage-induced phosphorylation of Chk1 is inhibited by caffeine,[210] suggesting that caffeine will also inhibit ATR-mediated phosphorylation of Chk1 in human cells. Caffeine also prevents the ionizing and UV radiation-induced phosphorylation of p53 on Ser-15, presumably by disrupting ATM and ATR function.[208] However, since caffeine preferentially sensitizes p53-deficient cells to DNA damage, the radiosensitizing effects of caffeine are most likely related to inhibition of ATM- and ATR-mediated activation of Chk2 and Chk1, respectively.

Pentoxifylline is a methylxanthine derivative that enhances the sensitivity of a wide variety of human tumor cells to DNA damaging agents.[211-213] While the precise mechanism of action of pentoxifylline has not been elucidated, several studies indicate that its ability to enhance chemosensitivity is a direct result of G2 checkpoint abrogation.[211] Like caffeine, pentoxifylline preferentially radiosensitizes p53-deficient cells.[213,214] When used in combination with cisplatin, thiotepa, carboplatin, or cyclophosphamide, pentoxifylline enhances the ability of these compounds to inhibit the growth of murine mammary tumors in vivo.[215,216] Pentoxifylline also inhibits the growth of human bladder tumors[217] and human lung tumors[212] in mice xenograft tumor models. These promising preclinical results have prompted the evaluation of pentoxifylline in several clinical trials for efficacy against lung[218] and cervical[219] cancer in combination therapy with genotoxic chemotherapeutics such as ionizing radiation and cisplatin. Another related methylxanthine, lisofylline, also abrogates G2 checkpoint function and sensitizes p53-deficient human tumor cells to ionizing radiation[216,220] and cisplatin.[221] Further, lisofylline is more effective than pentoxifylline at enhancing the sensitivity of murine mammary tumor cells to ionizing radiation.[216,220]

Staurosporine is a non-specific protein kinase inhibitor that can override DNA damage-induced G2 delay in response to ionizing radiation.[222] However, the cytotoxicity of staurosporine has limited its potential clinical efficacy, leading to the development of staurosporine analogs with improved specificity and reduced cytotoxicity.[223] One such staurosporine derivative, UCN-01, is also a potent abrogator of the G2 cell cycle checkpoint and increases the cytotoxic effect of DNA-damaging agents in human tumor cells.[224,225] UCN-01 significantly inhibits the growth of a variety of human tumors in mice xenograft tumor models[226,227] and is currently in Phase I clinical trials showing promising results.[228] Preclinical studies have provided many mechanistic insights to UCN-01 activity. Treatment of tumor cells with UCN-01 results in Wee1 inactivation and Cdc25C activation, although these are indirect effects of UCN-01 inhibition of upstream checkpoint kinases.[229] UCN-01 specifically inhibits Chk1, as the related Chk2 kinase and the upstream ATM kinase are refractory to inhibition by UCN-01.[230] UCN-01 also inhibits C-TAK1 in vitro, although the contribution of this kinase to phosphorylation of Cdc25C during G2 checkpoint activation has not been fully elucidated.[231] Interestingly, UCN-01 selectively ablates the G2 checkpoint in cancer cells with defective p53 function.[224] Since ablation of G2 checkpoint function by UCN-01 can occur in the absence of p53, signaling to substrates other than p53 must be sufficient for G2 override.

Another approach to disrupt G2 checkpoint is the use of cell permeable peptides that can block specific G2 signaling components. For example, Suganuma et al[232] engineered short peptides corresponding to amino acids 211-221 of human Cdc25C fused with the retroviral TAT protein.[232] The TAT protein allows these fusion proteins to permeabilize the cell membrane and accumulate in excess of the endogenous Cdc25C, thus blocking Chk1 and Chk2 kinase activity toward the endogenous Cdc25C. Human tumor cells treated with these Cdc25C peptides are sensitized to DNA damage due to defective G2 checkpoint response, suggesting that Chk1 and Chk2 are effective targets to mediate abrogation of the G2 checkpoint.[232] Evidence supporting this latter strategy is provided by the recent report of SB-2180708, a selective Chk1 inhibitor structurally related to staurosporine that disrupts the G2 checkpoint.[233] In the presence of SB-2180708, HeLa cells exposed to ionizing radiation or topoisomerase I inhibitors fail to undergo a G2 arrest.[233] Further, inhibition of Chk1 activity in HeLa cells enhanced the cytotoxic effects of genotoxic agents, thus supporting the validity of Chk1 as a target for G2 checkpoint override.[233]

The p38$^{\gamma}$ cascade is another potential target for the development of radiosensitizing agents. Activation of p38$^{\gamma}$ is required for G2 arrest in tumor cells and fibroblasts exposed to ionizing radiation and disruption of p38$^{\gamma}$-dependent signaling enhances the sensitivity of these cells to ionizing radiation.[145] Since p38$^{\gamma}$ is inactive under normal cellular growth conditions and elimination of p38$^{\gamma}$-dependent signaling does not alter normal cell cycle progression,[145] the molecules in this pathway are potential targets for the development of inhibitors that will

mediate increased sensitivity to radiation therapy. Thus, a further understanding of the mechanism by which p38g regulates G2 arrest may lead to the development of novel strategies for the improvement of radiation therapy.

While the majority of anticancer drugs activate the G2 checkpoint after genotoxic stress by directly targeting kinases involved in DNA damage signaling pathways, alternative compounds that regulate the G2 checkpoint exist. For example, histone deacetylase inhibitors trigger a G2 arrest in normal human cells; however, this G2 arrest fails to occur in a diverse range of human tumor cells and they undergo mitotic catastrophe.[234] These compounds block histone deacetylase activity, increasing the acetylation state of the chromatin, altering chromatin structure and regulation of gene expression. These inhibitors may represent a novel mechanism of G2 override in tumor cells in the absence of concurrent DNA damage.[235] Of note, histone deacetylase inhibitors upregulate p21 to mediate cell cycle arrest at the G1/S and G2/M transitions,[236,237] as p21-/- colon carcinoma cells are resistant to these compounds.[238] The anticancer potential of histone deacetylase inhibitors has been demonstrated in both in vitro[236,239] and in vivo[240] model systems and several histone deacetylase inhibitors are currently being used in clinical trials with promising early results.[241]

In addition to providing insight to mechanisms that result in enhanced clinical efficacy, a further understanding of G2 checkpoint function may also lead to the identification of the signaling pathways that mediate tumor cell chemoresistance. For example, overexpression of the receptor tyrosine kinase, ErbB2 (HER2/neu), results in paclitaxel resistance in breast cancers.[242] Yu et al[242] demonstrated that ErbB2 overexpression in breast cancer cells results in upregulation of p21, which binds cyclin B1/Cdc2 complexes and inhibits paclitaxel-mediated Cdc2 activation and mitotic entry.[242] It is hypothesized that the ErbB2-mediated G2 arrest inhibits the action of paclitaxel, which requires cell transition into mitosis and/or cyclin B1/Cdc2 activation.[243] Based on this latter study, it was hypothesized that the paclitaxel resistance of ErbB2-overexpressing tumors could be eliminated by downregulation of ErB2 function. In support of this hypothesis, Baselga et al[244] recently demonstrated that combinatorial use of paclitaxel and anti-HER2 antibodies results in significant growth inhibition of HER2 overexpressing human breast cancer xenograft tumors as compared to treatment with either agent alone.

Screens for New Compounds

In an effort to identify novel anticancer agents, including those which may abrogate G2 checkpoint function, the National Cancer Institute has utilized a panel of 60 human tumor cell lines in a drug screen to identify and characterize compounds with anticancer activity.[245] To date, 70,000 compounds have been tested in these cell lines and the results recorded in a database.[245,246] Of note, p53 gene mutations occur in a majority of the NCI drug screen cell lines, as 39 of 58 cell lines analyzed contain a mutant p53 sequence and have defective biochemical p53 activity.[247] Thus, many of the cell lines analyzed in the drug screen are predicted to have impaired G2 checkpoint maintenance following DNA damage. In support of this hypothesis, cell lines containing mutant p53 exhibit less growth inhibition in this screen than the wt p53 cell lines when treated with the majority of clinically used anticancer agents, including DNA cross-linking agents, antimetabolites, and topoisomerase I and II inhibitors.[247] This latter result suggests that disruption of the G2 checkpoint may enhance the clinical efficacy of anticancer reagents and exemplifies the need for the development of novel agents that might induce G2 checkpoint override. Amundson et al recently evaluated the basal expression levels of 10 transcripts from genes that participate in DNA damage signaling pathways in these same NCI cell lines and correlated this data with the sensitivity of the cells to a panel of 122 standard chemotherapy agents.[246] Further, cDNA microarray analyses have been used to assess gene expression profiles of these same 60 cancer cell lines in response to several standard chemotherapeutic drugs.[248] Grouping the cell lines by patterns of gene expression resulted in different relationships than those obtained by clustering the cell lines as a function of their

chemosensitivity.[248] Analyses such as these may provide valuable insight as to how the transcription of specific genes relates to drug sensitivity. Similar strategies can be utilized in the future to examine the patterns of gene expression after treatment of cells with novel anticancer agents.

Investigators are also developing high-throughput screens to identify G2 checkpoint inhibitors. Roberge et al[249] used MCF-7 breast cancer cells that express a dominant-negative mutant p53 in a high-throughput screen for compounds that override ionizing radiation-induced G2 arrest and allow entry into mitosis. The loss of wt p53 function in these cells eliminates G1 checkpoint function and causes the majority of cells to arrest at the G2 checkpoint after exposure to ionizing radiation. To identify compounds that can ablate G2 checkpoint function, the mutant MCF-7 cells were grown in 96-well plates, irradiated to induce G2 arrest, and then co-treated with nocodazole and various extracts from marine invertebrates.[249] In the presence of a compound that could override the IR-induced G2 arrest, the cells were trapped in mitosis by the presence of the microtubule inhibitor nocodazole. The plates were then rapidly screened by use of an antibody that recognizes a phosphorylated form of nucleolin present only in mitotic cells. The reported screening process of 1300 extracts was validated by the isolation of staurosporine, a previously described G2 checkpoint inhibitor.[249] The screen identified one novel G2 inhibitor, isogranulatimide, a structurally unique compound. Isogranulatimide shows only mild toxicity to cells when used alone; however, treatment of the MCF-7 cells expressing mutant p53 with ionizing radiation and isogranulatimide results in synergistic cytotoxicity.[249] The use of this type of assay to identify G2 checkpoint inhibitors should allow further isolation of novel compounds that override G2 arrest.

Genetic Approaches

Since the major signaling pathways and cell cycle checkpoints are conserved between yeast and mammalian cells, yeast model systems may be manipulated to determine the molecular mechanism of anticancer drugs and to identify novel molecular targets for rational drug design. In contrast to mammalian cells, yeast offer the unique advantage of simple and rapid genetic manipulations that, coupled with the availability of the S. *cerevisiae* genomic sequence and the ongoing *S. pombe* genome sequencing project,[250] makes this organism an attractive model in which to evaluate chemotherapeutic agents. The Seattle Project encompassed multiple approaches for the discovery of anticancer targets and drugs through use of yeast genomics.[251] In one approach, a panel of isogenic yeast strains, each having single or multiple mutations in pathways involved in DNA repair, cell cycle checkpoint function, or cell cycle regulation were generated and used to screen new and existing anticancer drugs.[251] In a separate effort to identify novel cellular pathways to target for anticancer drug discovery, Norman et al[252] utilized budding yeast to genetically select peptide inhibitors. In this latter approach, peptides were selected based on phenotypic analyses and genetic dissections of candidate target pathways were performed to identify putative targets of the inhibitors.[252]

An additional example of the power of yeast genomics is the ability to perform cDNA microarray analyses to determine how genome-wide expression can be modulated in cells exposed to anticancer compounds.[253,254] This methodology was recently exploited by Jelinsky and Samson, as they used DNA chip technology (with the 6,200 *S. cerevisiae* genes represented) to compare the transcription profiles of *S. cerevisiae* treated with an alkylating agent, methyl methanesulfonate, to those of untreated cells.[254] The potential for microarray analyses to integrate existing regulatory networks is exemplified by the recent use of this technology to link the DNA excision repair pathway of *S. cerevisiae* to proteasome-associated control elements.[255] Further, Hughes et al[256] recently generated a database of expression profiles corresponding to 300 diverse mutations and chemical treatments in *S. cerevisiae* and used this database to identify novel genes required for various cellular functions as well as to identify novel target genes of known compounds. Of note, subsequent findings indicate that many yeast mutants exhibit chromosome-wide aneuploidy as compared to isogenic parental

wt strains.[257] This observation has significant implications for interpreting whole-genome transcriptional expression profile data, particularly data obtained from malignant or immortalized cells that are known to be genetically unstable.[257] Genetic approaches such as those described here will further validate the signaling pathways used by current chemotherapies, identify compounds with preferential lethality to cells with defective checkpoint function, and reveal additional signaling pathways to target for new drug discovery.

Future Directions

A fundamental challenge in the development of anticancer agents is the identification of molecular differences between cancer cells and normal cells that can be targeted for chemotherapeutic intervention to preferentially eliminate cancer cells while minimizing the toxicity to normal tissues. The drug discovery process for cancer will continue to be transformed by the wealth of information generated by the genome projects across many organisms, both prokaryotic and eukaryotic. As our understanding of cell cycle regulation and checkpoints increases so will the number of signaling molecules and pathways that can be used as targets for rational drug and therapy design. The hope is that from a detailed understanding of these processes, more incisive, mechanism-based approaches to cancer treatment will evolve that exploit the molecular defects in human tumors. To achieve this goal, we need to continue to develop

(1) technologies to precisely define the checkpoint defects in individual tumors,
(2) panels of anticancer agents that target cells with defined genetic alterations, and
(3) treatment regimens that are tailored to the resulting cell cycle phenotype.

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Ectopic Expression of Wild-Type Bcl-2, not S70A Phosphomutant Bcl-2, Confers Increased Cell Survival after Transient Taxol Treatment

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Abstract

It is hypothesized that the ability of microtubule inhibitors such as paclitaxel (Taxol®) to induce Bcl-2 phosphorylation contributes to their clinical efficacy. RKO colon carcinoma cells were engineered to ectopically express either wild-type (WT) or a phosphomutant Bcl-2 (S70A), in which the serine 70 residue, known to be phosphorylated in Taxol-treated cells, has been mutated to alanine. Established xenograft tumors comprised of the WT and S70A derivative RKO cells, remained sensitive to Taxol-mediated growth inhibition *in vivo*. Two dimensional protein analyses of Bcl-2 showed that Bcl-2 was phosphorylated *in vivo*; although the levels and number of phosphoforms were minimal and did not vary with treatment nor with the Bcl-2 isoform expressed. To determine if WT or S70A Bcl-2 expression could prolong tumor cell survival after transient Taxol treatment, Vector control, WT Bcl-2 and S70A Bcl-2-expressing cells were exposed to Taxol in monolayer culture and subsequently assayed for their ability to grow in soft agar or establish tumors in athymic, nude mice. Ectopic expression of WT Bcl-2 increased cell survival and proliferation in these assays. The results indicate that expression of WT or S70A Bcl-2 did not affect sensitivity of established xenograft tumors to Taxol. However, increased expression of WT Bcl-2, but not S70A Bcl-2, could promote tumor cell survival and proliferation in an anchorage-independent manner after transient Taxol exposure.

1. Introduction

Tumorigenesis is a complex process that requires alteration of several cellular pathways including those that control proliferation and survival. These alterations can decrease cellular sensitivity to apoptotic stimuli and allow for tumor growth. Several proteins control the initiation and execution of apoptosis and the Bcl-2 family plays a key role in this process. The activity of many of the Bcl-2 family members is regulated by post-translational modification including protease cleavage, myristoylation, and phosphorylation [6,11,25,27] . Bcl-2 has been shown to be a phospho-protein but the effect this modification has on its function remains controversial [10,12,13,16,18,26].

Bcl-2 phosphorylation occurs in cells treated with chemotherapeutic agents that inhibit microtubule function such as paclitaxel (Taxol), vincristine and vinblastine [7,9]. In human T cells and in acute myeloblastic leukemia (AML) blast cells, Taxol-treatment increases phosphorylation of Bcl-2 on serine and threonine residues [12,26]. Several studies have shown that in microtubule inhibitor-treated cells, mutation of serine 70 to alanine decreases Bcl-2 phosphorylation to very low levels and additional mutations at serine 87 and threonine 69 significantly reduces Bcl-2 phosphorylation [1,8,15,20,26]. It has been proposed that phosphorylation of Bcl-2 inhibits its anti-apoptotic activity [10]. The expression of either S70A, S87A or S70,87A,T69A mutant Bcl-2 decreases the sensitivity of tumor cells to Taxol *in vitro* [20,26]. In addition, deletion of amino acids 32 to 80 within Bcl-2, which is often referred to as the loop domain, decreases Taxol-induced apoptosis in MDA-MB-231 breast cancer cells [20].

Previous studies, including ours, have demonstrated that Bcl-2 is phosphorylated in a cell cycle-dependent manner occurring normally when cells are in mitosis[14,19,26]. This finding accounts for the elevated levels of Bcl-2 phosphorylation observed when cells are treated with agents that arrest the cell cycle in mitosis, such as Taxol [7,9]. In the present study, we engineered RKO carcinoma cell lines to ectopically express either WT Bcl-2 or S70A Bcl-2 and assessed Bcl-2 phosphorylation patterns, cell proliferation and survival using various *in vivo* and *in vitro* assays.

2. Material and Methods

2.1 Generation, maintenance and flow cytometric analysis of cell lines

The S70A Bcl-2 cDNA was the kind gift of Carlo Croce (Kimmel Cancer Center, Philadelphia, PA); substitution of alanine for serine 70 was verified by sequencing [8]. The S70A cDNA was subcloned (Kpn I-NotI fragment) into the pCEP4 expression vector (Invitrogen). The generation of pCEP4-WT Bcl-2 has been described previously [17]. RKO-pCEP4 (RKO-Vector), RKO-WT Bcl-2 (RKO-WT) and RKO-S70A Bcl-2 (RKO-S70A) cell lines were engineered by lipofectamine transfection (Gibco BRL) of the RKO colorectal carcinoma cell line followed by selection in hygromycin B. Individual clones were expanded and the resultant cell lines were grown at 37 °C with 5% CO_2 in McCoys 5A (Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin and 200 μg/ml hygromycin B (Calbiochem). Where indicated, cells were treated with paclitaxel (Taxol; Sigma) suspended in DMSO. Flow cytometry was performed as previously described [19].

2.2 *Protein Analyses*

Protein lysates were prepared, quantified and processed by Western as previously described [19,24]. Membranes were incubated with antibodies against Bcl-2 (Clone 100, Oncogene Research Products, Calbiochem), MPM-2 (Mitotic protein monoclonal #2, Upstate Biotechnology), and Actin (I-19, Santa Cruz Biotechnology).

For the preparation of tumor lysates, tumors were minced in Kinase Lysis Buffer [KLB: 50 mM Tris pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 0.1% NP-40, 4 mM EDTA, 50 mM NaF, 0.1 mM NaV, 1 mM DTT and the protease inhibitors: antipain (10 μg/ml), leupeptin (10 μg/ml), pepstatin A (10 μg/ml), chymostatin (10 μg/ml), phenylmethylsulfonyl fluoride (50 μg/ml) (Sigma), and 4-(2-aminoethyl)-benzenesulfonylfluoride (200 μg/ml) (Calbiochem-Novabiochem Corp)] and

sonicated. To remove insoluble debris, the lysates were centrifuged twice at 14,000 *g*. Protein concentration of the extract was determined by the Bradford assay (Bio-Rad). 50 μg of protein lysate were analyzed by Western.

For the two dimensional (2D) gel analyses of Bcl-2 protein from cell lines, 150 μg of protein lysate (RKO-Vector) or 30 μg (RKO-WT and RKO-S70A) from control or Taxol-treated cells were prepared in a final volume of 550 μl or 450 μl of isoelectric focusing (IEF) sample buffer [9.5 M urea (Pharmacia), 2% NP-40, 2% ß-mercaptoethanol, 0.2% ampholytes pH 5-8 (Pharmacia), 0.001% bromophenol blue], respectively. An internal control permitted alignment of Bcl-2 phospho-forms. IEF was performed using the PROTEAN IEF system (Biorad) and 17 cm isoelectric strip gels pH 5-8 (Biorad). The isoelectric gels were actively rehydrated for 12 h at 50 volts in IEF sample buffer containing the protein lysate prior to focusing for 60,000 volt-hours. After IEF, gels were incubated for 15 min in equilibration buffer I [6 M urea, 2% SDS, 0.375 M Tris (pH 8.8), 20% glycerol, 130 mM DTT] and 15 min in equilibration buffer II [6 M urea, 2% SDS, 0.375 M Tris (pH 8.8), 20% glycerol, 135 mM iodoacetamide (Aldrich Chemical Company, Inc.)] prior to Western Analysis.

For 2D gel analyses of Bcl-2 protein isolated from tumor tissue, 200 μg of protein lysate (RKO-Vector) or 30 μg (RKO-WT and RKO-S70A) were prepared in a final volume of 550 μl or 450 μl of IEF sample buffer, respectively. The samples were processed as described above.

For phosphatase assay, protein lysates from control and Taxol-treated cells (Vector: 150 μg; WT & S70A: 30 μg) were incubated in 100 μl of 40 mM PIPES pH 6.0, 1 mM DTT for 10 min at 30 °C, followed by addition of potato acid phosphatase [Vector: 0.825 U; WT & S70A: 0.33 U (Boehringer Mannheim)]. The incubation was continued for 30 min at 30 °C (WT & S70A) or 60 min at 30 °C (Vector) with Vector lysates receiving additional enzyme (0.825 U) after the first 30 minutes of incubation. The phosphatase reactions were stopped by the addition of IEF sample buffer and the proteins were analyzed by 2D gel electrophoresis. As a control for non-specific proteolytic degradation, protein lysates were also incubated in the absence of phosphatase

under the same conditions and analyzed by Western (data not shown).

2.4 *Soft agar assays*

RKO-Vector, RKO-WT and RKO-S70A cells were Taxol-treated (50 nM) for 0 h, 8 h, 12 h, 16 h or 24 h prior to plating in soft agar, adherent cells were harvested and washed twice in McCoys 5A medium to remove the drug. Cells were plated at 1 x 10^4 per 35 mm dish in McCoy's 5A medium supplemented with 10% fetal bovine serum and 0.4% agar. Colonies were counted when untreated controls reached 50 to 100 microns in diameter using an Omnicon 3800 Tumor Colony Analysis system (BioLogics, Inc.).

2.5 *Xenograft growth assay*

RKO-Vector, RKO-WT, RKO-S70A cells (5 x 10^6) were resuspended in 0.2 ml of McCoy's 5A medium and injected subcutaneously into the shoulders and flanks of 4-6 week old female, athymic nude mice (Harlan Sprague Dawley). To control for differences in potential growth rate based on body location, the site of injection for each cell line was varied between mice. Three xenograft tumors, one derived from each cell line, were established per mouse, and were allowed to grow to a mean size of 100 mm^3. Mice with a similar range in tumor sizes were treated with either vehicle (PBS) or 5 mg/kg Taxol (Meade-Johnson) intraperitoneally for 5 consecutive days per week for three weeks. Tumor volume was determined by the equation $V=[L \times W^2] \times 0.5$, where V = volume, L = length, and W = width. On day 19, tumors were measured, animals euthanized, and tumors harvested for protein analyses (See Western Analysis and 2D gel electrophoresis).

In the pre-treatment studies, the RKO-Vector, RKO-WT or RKO-S70A cells were Taxol-treated (50 nM) for 16 h prior to injection, adherent cells were harvested, washed twice in McCoys 5A medium, resuspended (1 x 10^7 cells) in 0.2 ml of media and injected subcutaneously into the

shoulders and flanks of 4-6 week old female, athymic nude mice (Harlan Sprague Dawley) as described above. Flow cytometry confirmed the cells had a 4N DNA content at the time of injection (data not shown). Measurements of tumor volume were initiated 4 days after cell injection.

3. Results

To investigate the role of Bcl-2 serine 70 phosphorylation in cell cycle progression as well as Bcl-2 activity, we engineered cell lines in which different isoforms of Bcl-2 were stably expressed. The human colorectal carcinoma cell line, RKO, was transfected with expression vectors encoding either no protein (Vector), the WT Bcl-2 protein (WT) or the phosphomutant S70A protein (S70A). RKO cells were selected for these studies since they stably express ectopic Bcl-2 in contrast to several other epithelial cell lines [17]. Clones from each transfection were selected and expanded to cell lines. Several RKO-Vector, RKO-WT and RKO-S70A cell lines were established that shared the same properties and representative results from one cell line of each genotype are shown.

To verify expression of Bcl-2 in the derivative cell lines, protein lysates from RKO-Vector, RKO-WT and RKO-S70A cells were analyzed by Western. The relative levels of Bcl-2 expressed in the cell lines was determined using a Fluor-S Max imaging system and values were normalized to actin. Bcl-2 levels were 16- and 14-fold higher in the WT and S70A cell lines, respectively, as compared to the RKO-Vector cell line (Fig. 1A).

To analyze Bcl-2 phosphorylation patterns in relation to cell cycle position, the derivative cells were treated with Taxol (50 nM) for 8h, 24h, and 48h and Western and flow cytometric analyses performed (Fig. 1B). The levels of the mitotic-specific phosphoepitopes, recognized by the MPM-2 antibody, were also analyzed [4]. After 8 h of Taxol treatment, the three cell lines had predominantly a 4N DNA content by flow cytometric analysis and significant MPM-2 positivity by

Western, indicative of cells arrested in mitosis (Fig. 1B). All the derivative RKO cells lines remained arrested through 24 h of Taxol treatment; however, by 48 h of treatment, the majority of the RKO-Vector cell population was represented by a subdiploid peak on the flow histogram (Fig. 1B). The appearance of subdiploid DNA indicates that after the Taxol-induced mitotic arrest, a majority of the cells underwent apoptosis. In contrast, a significant fraction of the WT and S70A cells maintained a 4N DNA content, although these cells were no longer MPM-2 positive.

Previously, we have shown that Bcl-2 becomes phosphorylated during mitosis and Taxol-induced Bcl-2 phosphorylation is due to the accumulation of cells in this phase of the cell cycle [19]. In the current studies, a low level of phosphorylated Bcl-2 was present in asynchronously growing Vector cells (Fig. 1B, 0 h). The level of phosphorylated Bcl-2 increased after 8 h of Taxol treatment, and remained elevated through 24 h of treatment (Fig. 1B). In the WT cells, low levels of phosphorylated Bcl-2 were present in asynchronously growing cells (Fig. 1B, 0 h) and increased significantly after 8 h and 24 h of Taxol treatment. In the S70A cells, there was little, if any, detectable phosphorylated Bcl-2 in the asynchronously growing cells (Fig. 1B, 0h); however, after 8 h and 24 h of Taxol treatment, one slower migrating isoform of phosphorylated Bcl-2 was readily detected. Of note, after 8 h and 24 h of Taxol-treatment, Bcl-2 phosphorylation in all three cell lines paralleled MPM-2 reactivity. After 48 h of Taxol treatment, there was a loss of MPM-2 positivity in all three cell lines and Bcl-2 phosphorylation patterns were similar to those seen at 0 h. The reduction of Bcl-2 phosphorylation and loss of MPM-2 positivity is likely due to cell exit from mitosis and progression into G1 as observed in our previous studies [22,23]. The exit from mitosis can be accompanied by apoptosis or continued cell cycle progression and endoreduplication [22,23].

To test whether ectopic expression of WT or S70A Bcl-2 reduced Taxol chemosensitivity *in vivo,* xenograft tumors comprised of the three derivative RKO cells lines were established in athymic nude mice. When the tumors reached a mean size of 100 mm^3, the mice received either

vehicle (PBS), or 5 mg/kg Taxol by i.p. injection on 5 consecutive days/week for 3 weeks. The injected dose per mouse was approximately 40 μg Taxol/ml blood, assuming a blood volume of 2.5 ml for the mouse. This dose of Taxol is comparable to a standard 135 mg/m^2 human dose which is 47.7 μg Taxol/ml for a woman of average height and weight (5'6", 130 lbs). Representative results from two tumors of the six analyzed for each condition are presented in Figure 2A. The Bcl-2 status of the xenograft tumors did not affect sensitivity to Taxol. After three rounds of Taxol treatment, there was a uniform 4-fold reduction in the Vector, WT, and S70A tumor volume as compared to the tumors growing in the animals treated with PBS alone. These results demonstrate that overexpression of WT or S70A Bcl-2 did not reduce RKO sensitivity to Taxol when the cells were grown as established xenograft tumors *in vivo*.

To verify ectopic expression of WT and S70A Bcl-2 proteins *in vivo*, all the xenograft tumors were harvested 24 h after the final Taxol treatment on day 18 and protein lysates prepared for Western analyses (Fig. 2B). The cells from RKO-WT and RKO-S70A tumors maintained significantly elevated levels of Bcl-2 expression as compared to those from RKO-Vector tumors. The relative levels of Bcl-2 protein within the tumors were similar to those seen when the cell lines were grown in culture (Fig 1A). For example, Bcl-2 levels were 13- and 16-fold higher in the tumors comprised of RKO-WT and RKO-S70A cells, respectively, as compared to that present in RKO-Vector tumor cells [determined by normalizing values obtained from Fluor-S Max imaging to actin (data not shown)].

To determine if the Bcl-2 expressed within the tumors was differentially phosphorylated in a manner similar to that observed when the derivative tumor cell lines were exposed to Taxol in culture, two dimensional (2D) gel electrophoresis was performed followed by Western analysis for Bcl-2. This technique allowed definitive analysis of the phosphorylated forms of Bcl-2 within the tumor cells. To establish conditions for 2D analyses of Bcl-2, we first assessed the Bcl-2 isoforms present in the derivative RKO cell lines growing in culture. Monolayer cultures of the RKO cell

lines were treated with Taxol (100 nM) for 16 h and protein lysates were analyzed by 2D electrophoresis. Five-fold less protein lysate (30 μg) was analyzed from the RKO-WT and RKO-S70A cells as compared to the RKO-Vector line (150 μg) to allow clear detection of Bcl-2 isoforms.

In Taxol-treated RKO-Vector cells, there was an increase in the number of Bcl-2 isoforms compared to the untreated controls (Fig. 3A, Vector). The Bcl-2 isoform, designated P_1 migrated slower than P_0 in the second dimension and is representative of the slower migrating form of Bcl-2 seen by Western (Fig. 1B). In Taxol-treated RKO-WT cells, three isoforms were readily detectable, P_0, P_1, and P_2 (Fig. 3A, Taxol). Both P_1 and P_2 migrated slower than P_0 in the second dimension and are representative of two slower migrating forms identified by Western analysis (Fig. 1B). Different Bcl-2 isoforms were not readily detectable in lysates from Taxol-treated RKO-S70A cells. A low level of the P_1 isoform was apparent and these 2D results were consistent with Western analyses (Compare Fig. 3A and Fig. 1B).

To confirm that the multiple Bcl-2 species detected by 2D analysis were a result of phosphorylation, aliquots of control and Taxol-treated RKO-Vector, RKO-WT and RKO-S70A protein lysate were incubated with potato acid phosphatase prior to 2D analysis. Phosphatase treatment collapsed the phosphoforms to predominantly a single species of protein labeled P_0(Fig. 3B). Thus, sequential phosphoforms were indicated as P_1 and P_2. Of note, each of the various Bcl-2 phosphoforms likely comprise a mixture of phosphorylated Bcl-2 species with identical mass/charge ratios.

Having established conditions for the 2D analysis of Bcl-2 phosphoforms, protein lysates from the representative tumors presented in Figure 2 were analyzed. Only the P_0 isoform of Bcl-2 was readily detectable in RKO-Vector and RKO-S70A tumor lysates from control and Taxol-treated mice (Fig. 3C and E). In tumors derived from the RKO-WT cells, there were detectable P_1

Bcl-2 isoforms; however, the patterns of phosphorylation were relatively the same in tumors isolated from control and Taxol-treated mice (Fig. 3D).

The above results led us to hypothesize that the overexpression of either WT or S70A Bcl-2 was not sufficient to decrease sensitivity to Taxol or confer a growth advantage to pre-established xenograft tumors. However, the presence of overexpressed WT or S70A Bcl-2 in cells may promote the survival and ability of these cells to form *de novo* tumors after a transient Taxol exposure as compared to RKO-Vector cells. To test this hypothesis, we performed additional anchorage-independent growth assays with the derivative RKO cell lines after transient Taxol exposure.

In one set of assays, monolayer cultures of RKO-Vector, RKO-WT and RKO-S70A cells were treated with Taxol (50 nM) for either 0 h, 8 h, 12 h, 16 h, or 24 h. After Taxol exposure, adherent cells were harvested, washed twice, and plated in soft agar and colony formation assessed 7 days later. Treatment of the cell lines with Taxol for 8 h did not inhibit colony formation relative to the untreated controls (Fig. 4). However, after 12 h of Taxol exposure there was an ~2-fold decrease in the number of colonies formed with RKO-Vector cells, while the RKO-WT and RKO-S70A Bcl-2 cells formed the same or more colonies than untreated cells. For the RKO-Vector cells, as the time of Taxol-treatment increased, there was a concomitant decrease in colony number relative to the untreated control with a 30-fold decrease by 24 h of treatment (Fig. 4). In the Bcl-2 overexpressing cell lines, 16 h of Taxol treatment resulted in a 2-fold decrease in RKO-S70A colony number while the RKO-WT cells formed the same number of colonies as the 0 h controls (Fig. 4). However, after 24 h of Taxol pretreatment, the colony-forming ability of the WT cells was reduced by 4-fold and that of the S70A by 10-fold as compared to untreated cells. These results suggest that ectopic expression of WT and S70A Bcl-2 reduced the chemosensitivity of cells to Taxol and increased colony formation in soft agar after transient exposure to the drug.

To determine if ectopic expression of WT or S70A Bcl-2 affected the *in vivo* tumorigenicity of RKO cells that have been transiently exposed to Taxol, monolayer cultures of RKO-Vector,

RKO-WT and RKO-S70A cells were treated with Taxol for 16 h, adherent cells were harvested, washed twice, and injected into athymic, nude mice. In triplicate, all three cell types were implanted in the same mouse so animal-to-animal variation could be ruled-out. Further, the injection location of a given cell line was varied between the shoulders and flanks of mice to rule-out tumor growth variation based on anatomical position. Tumor formation was assessed for 84 days after implantation of the cells. Sixteen hours of Taxol pretreatment was chosen since it represented the shortest drug exposure time that showed differential results in the soft agar assays (Fig. 4). As a control, untreated cells were injected as described above and growth monitored. The results with untreated cells were similar to those presented in Figure 2A, with robust tumor formation by 21 days after implantation for all three cell lines (data not shown). In contrast, the cells that were treated with Taxol for 16 h prior to implantation had a significantly reduced ability to form robust xenograft tumors (Fig. 5). Only the WT Bcl-2 expressing cells were able to proliferate and generate tumors that persisted weeks after implantation. Three of the WT Bcl-2 tumors persisted in the mice for 7 weeks, and by 12 weeks two of the tumors had increased in volume to ~150 mm^3 (Fig. 5, WT). The results from the xenograft tumor and soft agar studies suggest that ectopic expression of WT Bcl-2, but not S70A Bcl-2, can confer survival and proliferation potential to cells *in vivo* after a transient Taxol exposure.

4. Discussion

In the present study, we examined the effect of WT or S70A Bcl-2 expression on cell cycle progression, cell proliferation and survival of RKO colorectal carcinoma cells after Taxol treatment. In the monolayer, cell culture-based assays, there was a notable difference in the cellular response to Taxol when comparing Vector control cells to those overexpressing Bcl-2. Flow cytometric analyses showed that after 48 h of Taxol treatment, the majority of the RKO-Vector cell population was represented by a large subdiploid DNA peak, while the RKO-WT and RKO-S70A cell

populations were largely comprised of cells arrested with a 4N DNA content. However, these Taxol sensitivity were not recapitulated *in vivo* with established xenograft tumors in athymic nude mice. Regardless of the levels of Bcl-2 expressed or whether it was WT or the phosphomutant form of the protein, the xenograft tumors were equally susceptible to Taxol-mediated growth inhibition. Further, 2D analysis of Bcl-2 phosphorylation patterns in protein lysates from tumors harvested on the final day of Taxol treatment did not reveal significant changes in Bcl-2 phosphorylation in any of the tumors. However, if the RKO derivative cell lines were pretreated with Taxol prior to plating in soft agar or injecting into athymic nude mice, the WT Bcl-2-expressing cells had an increased ability to grow in an anchorage-independent manner *in vitro* and to establish tumors *in vivo*.

In agreement with our results, other groups have shown that overexpression of WT Bcl-2 in cells decreases their sensitivity to Taxol-mediated apoptosis *in vitro*. For example, the expression of WT Bcl-2 in MCF-7 and MDA-MB-231 cells decreases the percentage of apoptotic cells in cultures treated with low concentrations of Taxol (1-100 nM for MCF-7 and 1-10 nM for MDA-MB-231 cells) [21]. Overexpression of WT Bcl-2 in HL60 cells decreases their susceptibility to apoptosis after Taxol-treatment (500 nM) [5]. In MDA-MB-231 cells or Jurkat cells, the expression of S70A Bcl-2 increased the viability of Taxol-treated cells when compared to cells that expressed either no Bcl-2 or the wild-type protein [20,26]. However, in the current study, the cells expressing S70A Bcl-2 did not have increased viability in our anchorage-independent growth assays as compared to those expressing WT Bcl-2.

Our 2D protein analyses show a Taxol-induced elevation in the number of Bcl-2 phosphoforms in RKO derivative cell lines growing in culture; although, differences in the number of Bcl-2 phosphoforms were not apparent in either xenograft tumor lysates prepared from PBS- or Taxol-treated athymic, nude mice. Several possibilities exist for why we did not detect a significant increase in the number of Bcl-2 phosphoforms in the xenograft tumors from Taxol-treated mice. It is possible that after Taxol-treatment, phosphorylated Bcl-2 in the xenograft tumor cells was

ubiquitinated and degraded as previously shown by Chadebech *et al.* in ovarian carcinoma cells [3], or proteolytically cleaved as shown by Blagosklonny et al. in HL60 cells [2]. However, Western analysis of the protein lysates from Taxol-treated cells growing *in vitro* or *in vivo* did not show a significant decrease in Bcl-2 protein levels or the appearance of a proteolytically cleaved, faster migrating form of the protein. This latter finding was observed even when we analyzed 200 μg of protein lysate from cells overexpressing 13-fold higher levels of Bcl-2. Another theory for why we were unable to detect multiple phosphorylated forms of Bcl-2 in the xenograft tumors, is that the low levels of Bcl-2 phosphorylation in tumor cells from Taxol-treated animals is due to the exit of the cells from mitosis and subsequent entry into G1 [22,23]. Stewart *et al.* have shown that loss of Bcl-2 phosphorylation and MPM-2 reactivity in Taxol-treated HCT116 colon carcinoma cells coincides with mitotic exit and biochemical reentry of the cells into G1 [23]. Immunohistochemical analysis of the xenograft tumors indicated a very low level of MPM-2 reactivity (CDS & JAP, unpublished results) indicating that the cells within the tumor were not in mitosis at the time of analysis, thus Bcl-2 would not be phosphorylated, as we have previously shown [19].

In the treatment of human cancer, long-term patient survival is, in part, dependent upon the prevention of recurrent disease. Micro-metastases often remain dormant for many years prior to detection as secondary tumors. It is apparent from the results of the current study that elevated expression of the anti-apoptotic protein Bcl-2 increases the resistance of cells to apoptosis after transient Taxol exposure. If inappropriate expression of Bcl-2 occurs in a very small percentage of an individual's tumor cells, the result could be increased resistance and subsequent cell survival leading to recurrent tumor formation.

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Fig. 1. Phosphorylation of ectopically expressed WT or Phosphomutant Bcl-2 in Taxol-treated RKO cells. (A) Bcl-2 and actin proteins from asynchronous RKO-Vector, RKO-WT and RKO-S70A cells were analyzed by Western, and quantified using the Fluor-S Max imaging system. *Fold increase*, the increase in Bcl-2 levels in cells that ectopically express either WT or S70A Bcl-2 relative to Vector cells, and normalized to actin. (B) Asynchronous RKO-Vector, RKO-WT and RKO-S70A cells were treated with Taxol (50 nM) for 0 h, 8 h, 24 h and 48 h and harvested for flow cytometric and protein analyses. To determine cell cycle position, DNA content was determined by flow cytometric analysis. Representative peaks of 2N, 4N and <2N DNA content are indicated on the histograms. All histograms have the same x- and y-axes. Levels of Bcl-2, Bax and MPM-2 positive epitopes in total cell lysates from control and Taxol-treated cells were analyzed by Western. The film exposure time for the WT-Bcl2 and S70A Bcl-2 Western signals was 1/30th that for the Vector control signal. Bcl-2 phospho-forms are indicated by arrows. Results are representative of three independent experiments.

Fig. 2. Effect of Taxol on RKO derivative cell line xenograft tumor growth. (A) RKO-Vector, RKO-WT, RKO-S70A cells were grown as xenograft tumors in athymic nude mice to a mean volume of 100 mm^3, and then mice were treated with either vehicle (PBS) or 5 mg/kg Taxol intraperitoneally for 5 consecutive days per week for three weeks. The experiment was performed twice with 3 mice per treatment group. Results shown are from 2 representative mice with subcutaneous tumors on the shoulders or flanks that received either PBS (C1, C2) or Taxol (T1, T2). Data are plotted as the tumor volume relative to initial volume at day 1 of treatment. (B) on day 19, xenograft tumors were harvested for protein analyses, and the levels of Bcl-2 in tumor lysates from the PBS (C1, C2) and Taxol-treated (T1, T2) mice presented in *A* were determined by Western.

Fig. 3. Effect of Taxol on Bcl-2 phosphorylation in RKO cells growing in monolayer culture and in xenograft tumors. (A) Total cell lysates from control (Con) and Taxol-treated (100 nM) RKO-Vector, RKO-WT and RKO-S70A cells were analyzed by 2D gel electrophoresis followed by Western analysis for Bcl-2 phospho-isoforms. (B) phosphatase-treated total cell lysates from control (Con) and Taxol-treated (100 nM) RKO-Vector, RKO-WT and RKO-S70A cells were analyzed as above. Lysates from xenograft tumors derived from RKO-Vector (C), RKO-WT (D) and RKO-S70A (E) grown in the mice treated with PBS (C1, C2) and Taxol (T1, T2) presented in Fig. 2 were analyzed as above. 2D Bcl-2 protein patterns were aligned according to the position of an internal standard and the different phosphoforms of Bcl-2 were designated P_0, P_1, and P_2. These various Bcl-2 phosphoforms (P_0, P_1, P_2) likely comprise a mixture of phosphorylated Bcl-2 species with identical mass/charge ratios.

Fig. 4. Effect of Taxol pretreatment on anchorage-independent growth of RKO cell lines. RKO-Vector, RKO-WT and RKO-S70A cell lines were Taxol-treated (50 nM) and at the indicated time points adherent cells were harvested, washed twice and plated at 1 x 10^4 per 35 mm dish in medium and 0.4% agar. Colonies were counted when controls reached 50-100 microns in diameter. Values are representative of two independent experiments carried out in quadruplicate. *% of Control,* represents (pretreated colonies/untreated control colonies) x 100.

Fig. 5. Effect of Taxol pretreatment on RKO derivative cell line xenograft tumor growth. RKO-Vector, RKO-WT or RKO-S70A cells were Taxol-treated (50 nM) for 16 h, adherent cells were harvested, washed twice, and 1 x 10^7 cells were injected subcutaneously into the shoulders and flanks of athymic nude mice. The results shown are the xenograft tumors established from 6 individual injections of pretreated cells per cell line. Measurements of tumor volume were initiated 4 days after injection of cells and plotted as mm^3.

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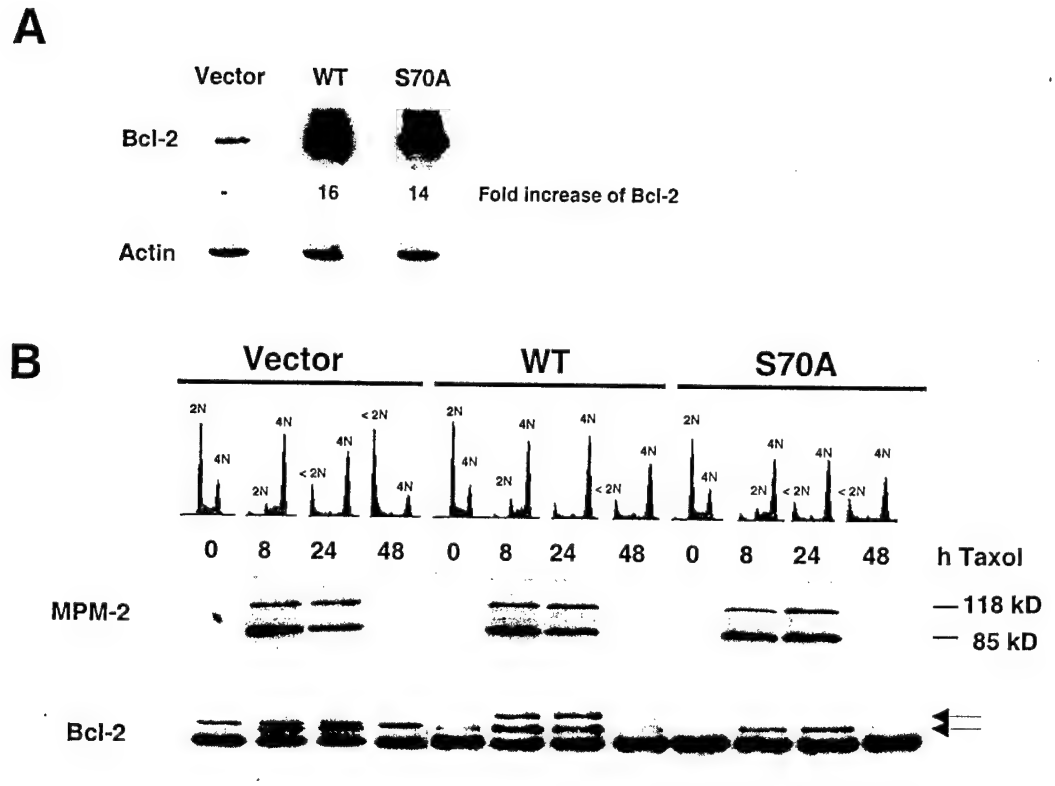

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Figure 1

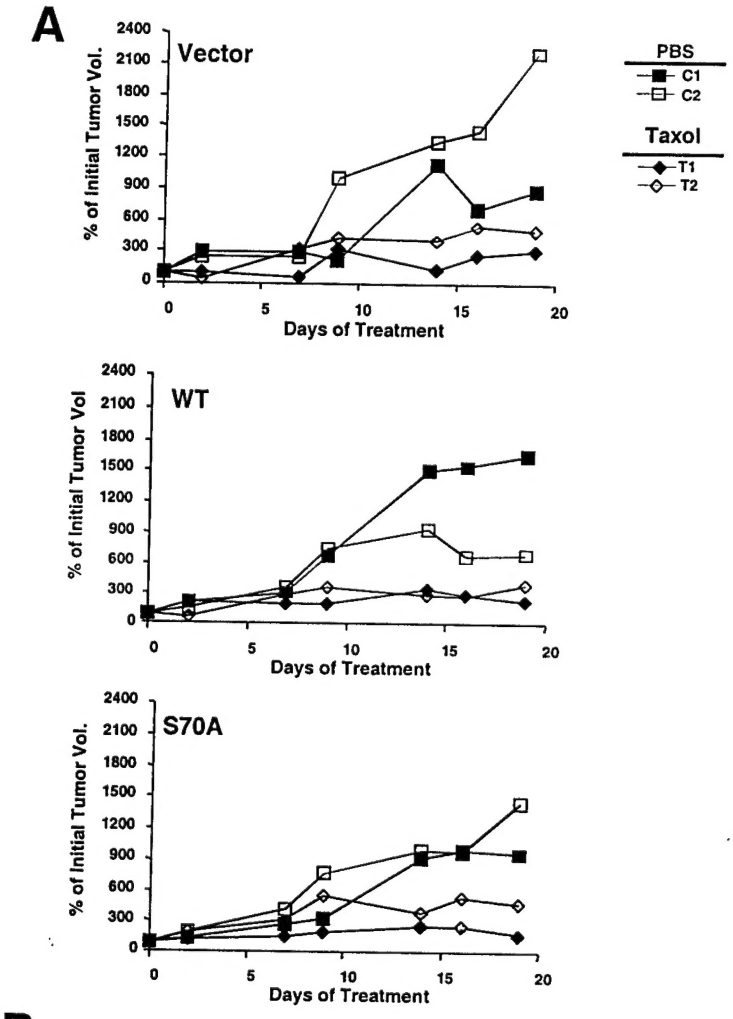

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Figure 2

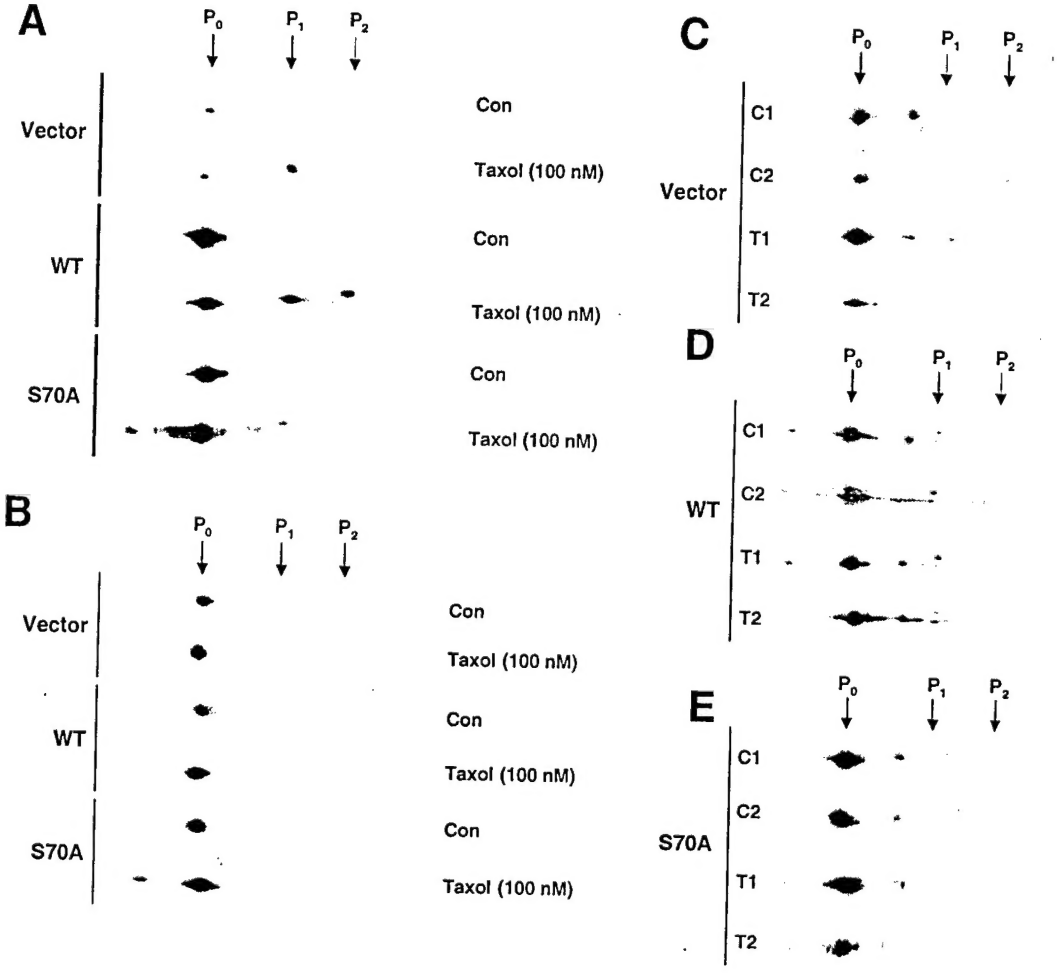

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Figure 3

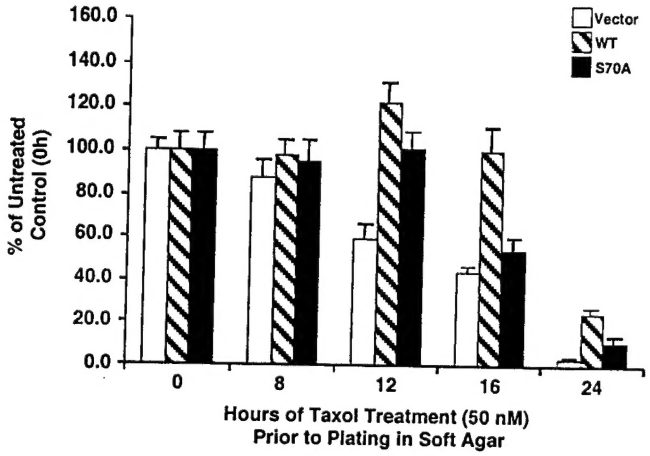

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Figure 4

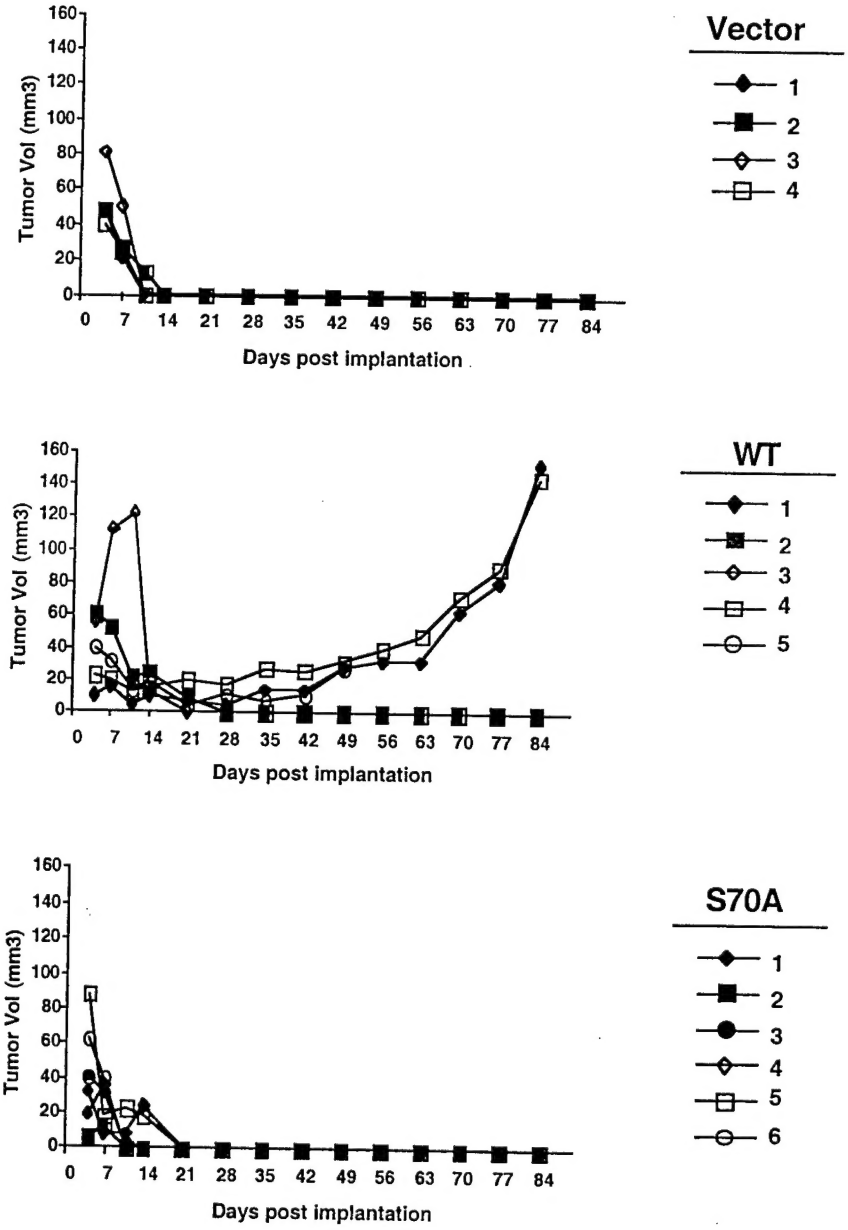

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Figure 5